REMARKS

Status of the Claims

Claims 1, 2, 5, 6, 7, 9, and 10 are pending.

As an initial matter, Applicants note that the Office Action states that claims 1, 2, 5, 7, 9, and 10 are pending and that claim 6 has been withdrawn. Applicants believe that this statement in the Office Action stems from Applicants' typographical error in the response filed June 23, 2003. Applicants inadvertently stated that claims 11-23 and 3, 5, 6, 8, and 9 were withdrawn because they were directed to a nonelected invention. Applicants previously elected claims in Group I (claims 1-10), directed to an antigenic peptide in response to a restriction requirement. Claim 6 is directed to a vaccine composition comprising an antigenic peptide having the subsequence of SEQ ID NO: 1 and 31. Thus, claim 6 is within the scope of the elected invention. Moreover, the Listing of the Claims accompanying the response filed on June 23, 2003 indicates that claim 6 was amended, not withdrawn.

Claims 1, 6, and 9 have been amended. More particularly, claims 1 and 6 have been amended for clarity to delete the recitation "selected from the group consisting of" and claim 9 has been amended for clarity to add the recitation "SEQ ID NO: 14"

The claims have been rejected, in various combinations, under 35 U.S.C. § 112, first paragraph, 35 U.S.C. § 112, second paragraph, and 35 U.S.C. § 101. Each of these rejections is addressed below.

The Invention

The present invention is directed to identification of peptide sequences associated with the long term survival of persons infected with HIV-1. The peptides (*i.e.*, epitope mimics or mimeotopes) were identified by screening peptide display libraries using sera from long term non-progressor HIV-1 infected individuals. The currently pending claims encompass the sequence X-KSSGKLIS-X which corresponds to SEQ ID NOS: 1 and 31 and to the epitope displayed on phage 195 (Fig. 2A). In some embodiments, the invention is directed to vaccines comprising the peptides.

Objection to Claim 1

The Examiner has correctly noted that the previous amendment to claim 1 added the recitation SEQ ID NO:31 and indicates that the recitation was not indicated as being newly added. Applicants note that SEQ ID NOS: 1 and 31 comprise the same nucleotide subsequence, *i.e.*, X-KSSGKLISL-X.

Rejection of Claims Under 35 U.S.C. § 112, second paragraph

Claims 1 and 10 are rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite.

Claim 1 has been amended in accordance with the Examiner's suggestion to delete the recitation "selected from the group consisting of." Accordingly, Applicants respectfully request withdrawal of this aspect of the rejection under 35 U.S.C. § 112, second paragraph.

Claim 10 has been rejected as allegedly unclear for the recitation "does not give rise to HIV-1 specific antibodies to more than twelve other antigenic determinants on HIV-1." In making the rejection, the Examiner alleges that the claim is indefinite because one of skill in the art could not know all of the existing sequences of HIV-1. Applicants respectfully traverse.

As set forth in MPEP §2173.03, definiteness of claim language is analyzed in light of (a) the content of the application disclosure; (b) the teachings of the prior art; and (c) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art.

Applicants respectfully assert that the claim does not require knowledge of all of the existing sequences of HIV-1. The claim requires: (1) that the antigenic peptide have a specific subsequence, *i.e.*, X-KSSGKLISL-X, and (2) that the ***subsequence*** comprises an antigenic determinant that does not give rise to HIV-1-specific antibodies to more than twelve other antigenic determinants on HIV-1. Thus, based on the particular amino acid sequence of the subsequence recited in the claims, one of skill in the art could easily determine peptides which

fall within the scope of the claim. Accordingly, Applicants respectfully request withdrawal of this aspect of the rejection under 35 U.S.C. § 112, second paragraph.

Rejection of Claims Under 35 U.S.C. § 101

Claims 5, 7, 9, and 10 are rejected under 35 U.S.C. § 101 as allegedly inoperative and lacking patentable utility. Applicants respectfully traverse.

In making the rejection, the Examiner indicates that the rejection of claims 1 and 2, directed to antigenic peptides, under 35 U.S.C. § 101 has been withdrawn. Applicants note that claims 5 and 10 are also directed to antigenic peptides and respectfully submit that the rejection of these claims under 35 U.S.C. § 101 should also be withdrawn. Moreover, as previously explained, the antigenic peptides of the invention have utility associated with diagnosis of HIV-1 infection as well as therapeutic utilities. For example, the peptides or antibodies to the peptides can be used as diagnostic reagents in immunoassays (*see, e.g.*, specification at page 29, lines 7-11). In addition, the peptides can be used *in vivo* to raise antibodies to particular epitopes (*see, id.*). Accordingly, Applicants respectfully submit that the claimed peptides possess a utility that offers an immediate benefit to the public and request that the rejection of claims 5 and 10 under 35 U.S.C. § 101 also be withdrawn.

In rejecting claims 7 and 9, directed to vaccine compositions comprising a peptide with an antigenic subsequence comprising KSSGKLISL, the Examiner alleges that the Applicants have not demonstrated that the claimed vaccines either elicit an anti-viral immune response or induce a protective immune response. Applicants respectfully traverse.

It is well settled that an invention must have "practical utility" to meet the utility requirements of the patent laws. As noted by the Courts: "practical utility" is a shorthand way of attributing 'real-world' value to claimed subject matter. In other words, one skilled in the art can use a claimed discovery in a manner which provides some immediate benefit to the public." (*see, e.g.*, MPEP § 2107.03 III, citing *Nelson v. Bower and Crossley* 206 USPQ 881 (CCPA 1980)). As set forth in MPEP § 2107.03 III, it is well established that as long as there is a reasonable correlation between the data presented to show utility and the asserted utility, including data from *in vitro* assays or data from animal models, the requirements of § 101 are met. In the case of claims covering therapeutic methods, it is not proper to require actual

evidence of from studies in humans to support utility or to require evidence regarding the degree of effectiveness of the method. (MPEP §2107.03 IV).

As previously explained, the specification provides actual working examples demonstrating that administration of peptides comprising the sequence set forth in SEQ ID NOS: 1 and 31 to macaques and mice leads to (1) a specific antibody response in macaques (*see, e.g.*, page 36, line 6 to page 37, line 7); and (2) development of neutralizing antibodies against HIV-1 in mice (*see, e.g.*, specification at page 34, line 27 to page 36, line 4). These working examples provide reasonable evidence that the claimed vaccine compositions are useful for, *inter alia*, diagnosis and therapy of HIV-1 infection.

In addition to the experiments set forth in the specification, the accompanying Declaration under 37 C.F.R. § 1.132 by Dr. Giuseppe Scala, one of skill in the art, further describes experiments that confirm that vaccine compositions comprising the claimed peptides induce a protective response against HIV in rhesus macaques. As Dr. Scala explains, macaques are an art accepted model for studies of HIV-1 infection (*see*, Declaration ¶¶ 6-7). Macaques vaccinated with peptides having a sequence equivalent to SEQ ID NOS: 1 and 31 exhibited low to undetectable plasma HIV loads and did not develop symptoms associated with SHIV-1 infection following viral challenge (*see*, Declaration ¶ 6). In contrast, control macaques exhibited high levels of plasma HIV loads and multiple symptoms associated with SHIV-1 infection following viral challenge (*see*, Declaration ¶ 6). The experiments described by Dr. Scala unequivocally demonstrate that the vaccine compositions comprising the claimed peptides actually induce a protective response *in vivo*.

Those of skill in the art appreciate that findings in macaques are well accepted in the art as extendible to other organisms, including humans. First, as set forth in the Declaration of Dr. Scala, it is well known to those of skill in the art that results from studies in macaques are reasonably predictive of similar results in other primates, including humans. In particular, infection of macaques with SHIV as described in Chen *et al.*, has been validated as a model of HIV-1 infection in humans (*see*, Declaration ¶7). Finally, Malenbaum *et al.*, *J. Virol.* 75(19):9287-96 (2001) (copy attached as Exhibit C) describes experiments comparing the immune response of humans and macaques to HIV-env proteins. Malenbaum *et al.* found that

the ability of the macaque immune system to recognize HIV-1 gp120 is comparable to that of humans and concludes that "data on the relative efficacies of HIV-1 env-based vaccines generated in the SHIV-macaque model will be applicable to the human setting" (*see*, Malenbaum *et al.*, page 9294, col. 1, lines 19-25). Thus, one of skill in the art would appreciate that results demonstrating that the vaccine compositions comprising the claimed peptides actually induce a protective response *in vivo* in macaques are reasonably predictive of similar results in other organisms, including humans.

In view of the foregoing, Applicants submit that the claimed peptides and vaccine compositions have utility that exceeds the minimal requirements of 35 U.S.C. § 101. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 101.

Rejection of Claims Under 35 U.S.C. § 112, first paragraph

Claims 1, 2, 5, 7, and 9 are rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement. Applicants respectfully traverse. In making the rejection, the Examiner acknowledges that the specification is enabling for an antigenic composition, but alleges that the specification does not enable a vaccine that protects against HIV-1.

A particular claim is enabled by the disclosure in an application if the disclosure, at the time of filing, contains sufficient information so as to enable one of skill in the art to make and use the claimed invention without undue experimentation. *See, e.g., In re Wands*, 8 USPQ2d, 1400 (Fed. Cir. 1988), or MPEP §2164.01. Moreover, as set forth in MPEP § 2164.02, "[a] rigorous or an invariable exact correlation is not required" between a particular model and a specific condition.

As an initial matter, Applicants note that claims 1, 2, and 5 are directed to antigenic peptides. Accordingly, Applicants respectfully assert that in view of the Examiner's acknowledgement that the specification is enabling for antigenic compositions, the rejection under 35 U.S.C. § 112, first paragraph does not apply to claims 1, 2, and 5. Moreover, the specification fully enables the antigenic peptides as disclosed and claimed in the present application. For example, antigenic peptides comprising the sequence set forth in SEQ ID NOS: 1 and 31 are fully described in the specification at, *e.g.*, page 2, lines 5-6. In addition, the

specification at page 12, line 4 to page 16, line 4 describes a variety of methods for preparing the peptides of the invention including, *e.g.*, chemical synthesis and recombinant DNA technology. Thus, as the Examiner has acknowledge, the specification fully enables the claimed antigenic peptides and Applicants respectfully request withdrawal of this aspect of the rejection under 35 U.S.C. § 112, first paragraph.

With respect to the rejection of claims 7 and 9 under 35 U.S.C. § 112, first paragraph, Applicants respectfully assert that the specification provides sufficient guidance for one of skill in the art to make the claimed vaccines and to use the vaccines to generate an immune response against HIV-1. As explained above, there is ample support in the specification for peptides comprising SEQ ID NOS: 1 and 31 (*see, e.g.*, page 1, line 30) and methods of synthesizing the peptides (*see, e.g.*, page 12, line 5 to page 13, line19). Similarly, methods of making and administering vaccine formulations are well known in the art and are set forth in detail in the specification at, *e.g.*, page 2, lines 3-6; page 21, line 7 to page 22, line 24 and page 22, line 27 to page 24, line 30. In addition the specification describes experiments demonstrating that administration of peptides comprising the sequence set forth in SEQ ID NOS: 1 and 31 to macaques and mice leads to (1) a specific antibody response in macaques (see, e.g., page 36, line 6 to page 37, line 7); and (2) development of neutralizing antibodies against HIV-1 in mice (see, e.g., specification at page 34, line 27 to page 36, line 4). Finally, Dr. Scala has provided additional evidence confirming that administration of peptides comprising the sequence set forth in SEQ ID NOS: 1 and 31 to macaques leads to a protective response against SHIV-1 infection, an art accepted model for HIV-1 (*see, e.g.*, Declaration ¶6).

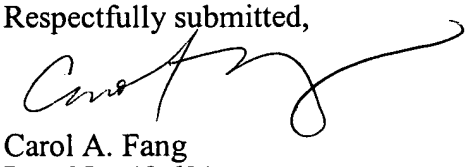
Applicants respectfully submit that in view of the guidance in the specification and what is known in the art, one of skill in the art would be able to use the claimed antigenic peptides and vaccines to induce an immune response against HIV-1. Accordingly, Applicants request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at 415-576-0200.

Respectfully submitted,



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PATENT
Attorney Docket No.: 015280-386200US
Client Ref. No.: E-070-99/1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner: Jeffrey J. Stucker
SCALA *et al.*	Art Unit: 1648
Application No.: 09/869,003	DECLARATION UNDER 37 C.F.R. § 1.132
Filed: September 25, 2001	
For: NOVEL HIV RELATED PEPTIDES	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Giuseppe Scala, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

2. I hold a M.D. (1975) from the University of Naples, (Naples, Italy). I am presently a Professor of Biochemistry and Molecular Biology at University of Catanzaro (Catanzaro, Italy) and University "Federico II," (Naples, Italy).

My field of expertise is Virology and Immunology, in particular, HIV pathogenesis and vaccine development. I have authored over 60 publications in the field of Immunology, HIV pathogenesis and vaccine development. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A.

3. The present invention is directed to identification of peptide sequences associated with the long term survival of persons infected with HIV-1. The

peptides (*i.e.*, epitope mimics or mimotopes) were identified by screening phage-displayed peptide libraries using sera from long term non-progressor HIV-1 infected individuals. The currently pending claims encompass the sequence X-KSSGKLIS-X which corresponds to SEQ ID NOS: 1 and 31 and to the epitope displayed on phage 195 (Fig. 2A). In some embodiments, the invention is directed to vaccines comprising the peptides.

4. I have read and am familiar with the contents of the subject patent application. I have also read the Office Action received from the United States Patent and Trademark Office dated December 9, 2003. It is my understanding that the Examiner is concerned that the claimed compositions and methods (1) lack utility and (2) are not enabled. Specifically, the Examiner alleges that the claimed vaccines lack utility because they do not induce a protective immune response and that the claimed antigenic peptides and vaccines are not enabled because Applicants have allegedly not taught how to use the peptides to induce an anti-viral effect.

5. This declaration provides additional experiments that demonstrate that the claimed vaccines comprising mimeotope peptides induce a protective immune response, results that are reasonably predictive that a protective immune response would be generated in humans using the same vaccine. The data was published in Chen *et al.*, *Nature Med.* 7(11):1225 (2001) (Copy attached as Exhibit B)

6. The experiments described in Chen *et al.* were conducted under my supervision. The models used in carrying out the experiments in Chen *et al.* are art accepted models for studying HIV-1 infection. Chen *et al* describe experiments in which rhesus macaques were vaccinated with HIV-1 mimotopes, one of which was KSSGKLISL (equivalent to SEQ ID NOS: 1 and 31 of the instant application). The animals were challenged with SHIV, a chimeric R5-tropic simian/human immunodeficiency virus. Macaques that were immunized with the HIV-1 mimotopes exhibited low to undetectable plasma SHIV loads and did not develop symptoms associated with HIV-1 infection following SHIV challenge, *i.e.*, there was no progression

to AIDS-like illness in vaccinated macaques. In contrast, naïve and mock-immunized macaques exhibited high levels of plasma SHIV loads and multiple symptoms associated with HIV-1 infection and AIDS-like illness including, *e.g.*, untreatable anemia, fever, multiple infections, hemorrhagic diarrhea, and weight loss following SHIV challenge. A comparison of the plasma SHIV loads in native, mock-immunized, and mimotope-immunized macaques is depicted in Figure 2 of Chen *et al.* Text explicitly describing the protective effects of the vaccination with HIV-1 mimotopes can be found at page 1229, col. 2, lines 22-30.

7. One of skill in the art would appreciate that findings in macaques described in ¶ 6 above, can be extended to other organisms, including humans. For example, as explained in Chen *et al.*, the results in the macaques are consistent with results seen in human studies in which low viral loads in plasma are associated with a good prognosis (*see, e.g.*, page 1229, col. 2, lines 29-32). Moreover, those of skill in the art appreciate that results from studies using the macaque model of HIV infection are reasonably predictive of similar results in other primates, including humans. Indeed, infection of rhesus macaques with recombinant SHIVs is a validated model of HIV-1 infection in humans.

8. In view of the foregoing, it is my scientific opinion that the claimed vaccines have utility and do induce a protective immune response against HIV. Therefore, contrary to the Examiner's allegations, the claimed vaccines have utility and the claimed antigenic peptides and vaccines are enabled.

Dated: May 3, 2006 By: _____
Giuseppe Scala, Ph.D.

Giuseppe Scala

Place and date of birth: Naples-Italy, February 9, 1951

Education

1974 M.D., University of Naples, Naples, Italy.
1979 Ph.D in Internal Medicine, Catholic University, Rome, Italy.
1985 Ph.D. in Clinical Immunology, University of Florence, Florence, Italy.

Employment

1975-94 Assistant professor, University of Naples. Naples-Italy.
1994-present: Full professor of Biochemistry and Molecular Biology, University of Catanzaro *Magna Graecia*, Catanzaro-Italy.

Stages

1981-1984 Fogarthy fellow, National cancer institute, National Institutes of Health, Bethesda, MD-USA
1988. 1988 Short-term fellowship, Biogene Biotechnology, Geneva.
1996-2001 Visiting Professor, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD-USA

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Protection of rhesus macaques against disease progression from pathogenic SHIV-89.6PD by vaccination with phage-displayed HIV-1 epitopes

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The antigenic polymorphism of HIV-1 is a major obstacle in developing an effective vaccine. Accordingly, we screened random peptide libraries (RPLs) displayed on phage with antibodies from HIV-infected individuals and identified an array of HIV-specific epitopes that behave as antigenic mimics of conformational epitopes of gp120 and gp41 proteins. We report that the selected epitopes are shared by a collection of HIV-1 isolates of clades A–F. The phage-borne epitopes are immunogenic in rhesus macaques, where they elicit envelope-specific antibody responses. Upon intravenous challenge with 60 MID_{50} of pathogenic SHIV-89.6PD, all monkeys became infected; however, in contrast to the naive and mock-immunized monkeys, four of five mimotope-immunized monkeys experienced lower levels of peak viremia, followed by viral set points of undetectable or transient levels of viremia and a mild decline of $CD4^+$ T cells, and were protected from progression to AIDS-like illness. These results provide a new approach to the design of broadly protective HIV-1 vaccines.

A number of studies in animal models have shown a protective role of antibodies (Abs) against acute infection with HIV-1 and simian HIV (SHIV) strains. Adoptive immunotherapy with anti-HIV envelope monoclonal antibodies (mAbs) and HIV-specific immunoglobulins (Igs) has resulted in protection from HIV-1 and SHIV challenge in nonhuman primates. Sterilizing immunity was obtained by passive transfer of monoclonal antibodies and serum immunoglobulins (Igs) from HIV-1 infected subjects or monkeys chronically infected with HIV-1 isolates[1-4]. These results show that protective antibodies are elicited in the course of natural HIV/SHIV infection and indicate that an effective vaccine should elicit, among other effects, an antibody response to HIV-1 envelope proteins[5]. Efforts to induce a broadly protective antibody response by immunization with gp120 protein or peptides have been frustrated, however, by the high antigenic polymorphism of HIV-1 envelope, a consequence of the high rate of mutation in the constant and variable regions of HIV-1 envelope protein[6] and the complex envelope structure of gp120 as an oligomer associated with gp41 (refs. 7,8). The degree of antigenic polymorphism is increased by the continuous *in vivo* evolution of the virus as a result of Ab affinity maturation and viral escape[9], and is compounded by interclade recombinations and by the emergence of highly divergent new viral isolates[10]. Thus, the antigenic polymorphism of HIV-1 is a major obstacle in developing an effective vaccine. In this regard, although monkeys immunized with the envelope of a given HIV-1 isolate might be protected against a subsequent challenge with a viral strain carrying a homologous envelope, little or no protection is observed in monkeys challenged with heterologous viruses harboring a different gp160 (ref. 11). This lack of significant cross-protection raises further concerns about the capacity of envelope-based vaccines to afford substantial protection against field isolates[12,13]. These difficulties could be overcome by identifying pools of epitopes shared by different HIV-1 clades and quasispecies and using them as immunogens to induce a broad antibody response.

Combinatorial peptide libraries express a large collection of peptide sequences (10^8 or more) that mimic linear or conformational epitopes of folded protein domains, and even carbohydrate structures that contribute to immunological escape[14-16]. Such libraries offer a good method to manage the complexity of the HIV-1 epitope repertoire by allowing the selection of HIV-specific epitopes shared by different HIV clades and quasispecies. We screened two random peptide libraries (RPL) using serum antibodies from subjects infected with HIV-1 clade B viruses and identified a pool of phage-displayed peptides that function as antigenic and immunogenic mimics of discrete domains of the HIV-1 envelope[17]. Phage-displayed peptides can be expressed in multiple copies (up to 2,700 copies when inserted in the N-terminal region of the pVIII coat protein of filamentous bacteriophage fd) and are able to induce specific Ab responses in immunized mice[18,19]. Furthermore, bacteriophage promote rapid

immunoglobulin class switching both in mouse and human subjects[18,20] and are well tolerated when injected in immunocompromised individuals, including those infected with HIV-1 (ref. 20). The phage-borne peptides seem to be processed intracellularly and presented in the context of MHC class I and II to elicit both cytolytic and T helper activity[21]. HIV-specific mimotopes are immunogenic in mice, where they elicit an antibody response that neutralized HIV-1 isolates in an *in vitro* infection assay[17]. These results indicate that the selected phage-displayed epitopes might induce a protective immune response when injected in nonhuman primates. To test this possibility, we immunized rhesus macaques with a selected pool of phage-displayed epitopes and challenged them with pathogenic SHIV-89.6PD. We report that immunizing monkeys with phage-borne epitopes resulted in reduced levels of viremia and protected the monkeys from progression to AIDS-like disease.

Immunization of rhesus macaques with phage-displayed epitopes

By screening RPLs with antibodies of HIV-infected individuals, we identified a series of phage-displayed epitopes that behaved as antigenic mimotopes of discrete regions of HIV-1 gp120 and gp41[17]. In particular, epitope p195 shares sequence homology with the gp120 V1 region (residues 112–120) of HIV-1–U16374, a primary isolate from an acute HIV seroconverter[22]; the p217 sequence matches the C2 region (residues 198–205) of HIV-1–U11607, a primary isolate from an AIDS patient[23]. These regions correspond to the first α helix and the third β sheet of the gp120 crystal structure, respectively, and are immunologically accessible by selected monoclonal antibodies as deduced by X-ray crystallography structure[24,25]. Epitope 197 mapped to a region of gp41 (residues 599–608) of the primary HIV-1 isolate HIVANT70 (ref. 26); this region is conserved among primary isolates of subtypes A–G and defines a disulfide-bonded structure recognized by a human monoclonal antibody[27] (Table 1). No obvious sequence homology with HIV proteins was detected in p287 and p335. However, previous studies indicated that these epitopes are antigenic mimics of gp160 epitopes[17]. When sera from individuals from diverse geographic regions who were documented to be infected with different HIV-1 clades (A–F) were analyzed for their ability to recognize the phage-displayed epitopes, a considerable degree of shared reactivity was seen (Table 2).

As antigenic mimics of HIV-1 envelope, the phage-borne epitopes would be expected to bind to antibodies from SHIV-infected monkeys. We tested this possibility by analyzing the antibody reactivity of monkeys infected with a series of SHIV strains carrying envelope proteins from different primary isolates. As shown in Table 3, antibodies from SHIV-infected monkeys reacted strongly with the pool of HIV-1 mimotopes, whereas antibodies from SIVmac239-infected monkeys did not. This study included rhesus monkeys infected with pathogenic SHIV-89.6PD that developed detectable amounts of anti-envelope antibodies (Table 3). Thus, by screening RPLs with HIV-1 antibodies, we identified an array of epitopes shared by a substantial percentage of primary isolates of different subtypes. These results are consistent with other studies in which peptide pools defined specific HIV-1 immunotypes shared by HIV-1 isolates of different subtypes[28].

As antigenic mimotopes of SHIV isolates, the selected epitopes are expected to induce an immune response to SHIV envelope and might prevent or inhibit SHIV infection when injected in nonhuman primates. We tested this possibility by immunizing a group of five rhesus macaques with a pool of five phagotopes (195, 197, 217, 287 and 335; 4×10^{13} physical particles each) in QS21 adjuvant; a second group of four rhesus macaques received equal amounts of wild-type phage in QS21 (2×10^{14} physical particles for each monkey) on the same immunization schedule (Fig. 1*a*). These epitopes were selected for their broad reactivity against antibodies from SHIV-infected monkeys (Table 3) and for their immunogenicity in mice[17]. We detected epitope-specific antibodies in monkeys 481, 485, 490 and 493 after the second mimotope immunization, and increasing levels thereafter. Monkey 480 had little epitope-specific Ab. We detected substantial amounts of Ab to oligomeric gp140-89.6 after the third immunization in all the immunized monkeys except monkey 480 (Web Fig. A). At the end of the immunization schedule, four phagotope-immunized monkeys (481, 485, 490 and 493) had raised substantial amounts of antibodies specific to each of the injected epitopes, with end-point titers ranging from 800 to

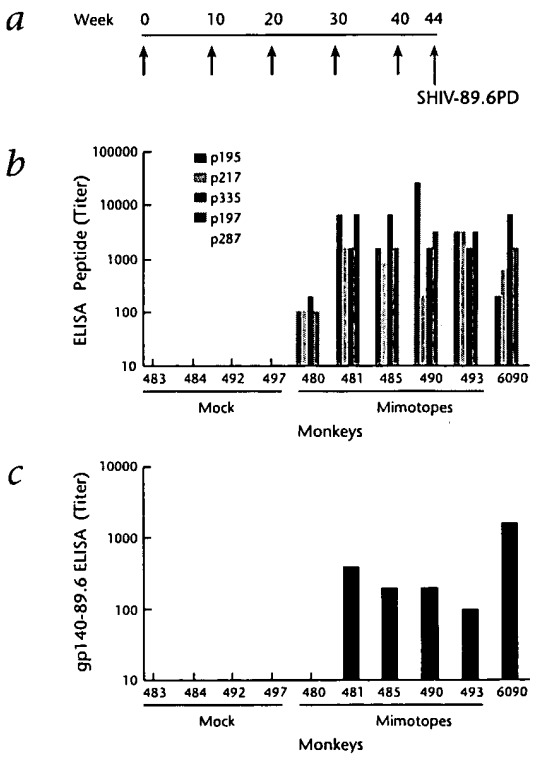

Fig. 1 Vaccine schedule and prechallenge antibody responses. ***a***, Immunization and challenge schedule. A pool of 5 epitopes (p195, p197, p217, p287 and p335) was injected into each quadricep of rhesus macaques 480, 481, 485, 490 and 493 (4×10^{13} physical particles each) with 100 μg QS21 adjuvant. In parallel, rhesus macaques 483, 484, 492 and 497 were immunized intramuscularly with f.11.1 wild-type phage (2×10^{14} physical particles each) with 100 μg QS21 adjuvant. Monkeys were immunized as shown at weeks 0, 10, 20, 30 and 40. ***b***, Antibody responses to the HIV-specific epitopes were determined by using short peptides whose primary sequence was engineered to function as surrogate mimotopes of the phage-displayed epitopes (see Methods). ELISA reactivities with linear dilutions of monkey plasma were assayed as described[42]. ***c***, Antibody responses to HIV-1 envelope were determined in ELISA by using a purified preparation of oligomeric gp140 (89.6 strain) as reported[42]. Panels *b* and *c* show the results from monkey plasma collected two weeks after the last immunization.

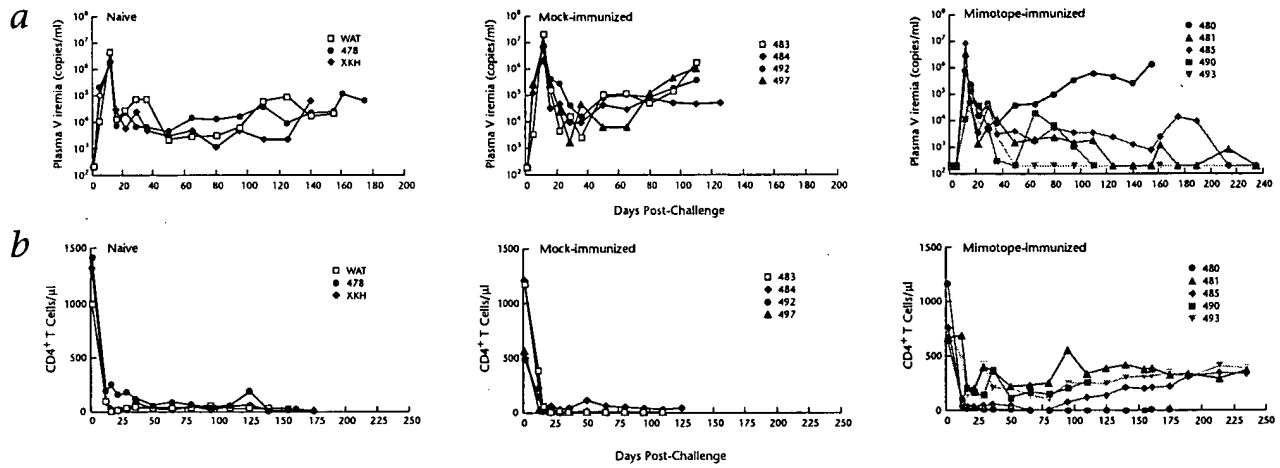

Fig. 2 Post-challenge levels of viremia and CD4⁺ T lymphocytes. Immunization and challenge schedule was as in Fig. 1*a*. ***a***, Post-challenge viral loads. 4 wk after the last immunization, a group of 3 naive rhesus macaques together with the group of monkeys injected with wild-type phage (mock-immunized) and the group of HIV mimotope-immunized monkeys (mimotope-immunized) were challenged by i.v. injection with 60 monkey infectious doses (MID_{50}) of cell-free SHIV-89.6PD. Plasma viremia was determined by branched DNA amplification with a detection limit of 200 copies per ml as reported[31]. ***b***, Post-challenge levels of CD4⁺ T lymphocytes. Peripheral CD4⁺ T cells were determined by multiplying the absolute lymphocyte counts by the percentages of CD3⁺CD4⁺ T cells detected by FACS analysis. Consistent results were obtained by determining the percentages of CD2⁺CD4⁺ and CD29⁺CD4⁺. No significant differences were observed in the case of CD3⁺CD8⁺ and CD2⁺CD20⁺ (data not shown).

24,000 (Fig. 1*b*). These titers were comparable to the peptide binding of serum 6090, obtained from a patient with good control of viremia, which was used in the initial screening of the RPLs (ref. 17).

Next, we tested the phagotope-immunized monkeys for antibody responses to HIV-1 envelope proteins. Four monkeys (481, 485, 490 and 493) that expressed high titers of antibodies to peptides tested positive in ELISA for antibody to HIV-gp140-89.6 (Fig. 1*c*). This reactivity was lower than that of serum 6090, which probably includes numerous antibody specificities. No ELISA reactivity was detected in the case of monkey 480. To assess whether antibodies to the immunodominant gp120 V3 loop could be elicited by mimotope immunization, we tested prechallenge plasma from monkey 493 for antibodies to peptide sequence corresponding to the V3 sequence of 89.6P. We did not detect antibodies to V3. In addition, increasing concentrations of V3 peptides did not interfere with the binding of plasma antibodies to the single epitopes, whereas binding was efficiently displaced by similar concentrations of peptides 195, 197, 217, 287 and 335 (Web Fig. B).

Viral challenge with SHIV-89.6PD

We next determined whether the mimotope-immunized monkeys are protected from viral challenge. We chose as a challenge virus SHIV 89.6PD, which was derived from cloned SHIV-89.6, carries a R5/X4 envelope and acquired pathogenic properties after *in vivo* passages; it consists of a swarm of several quasispecies and induces a rapid decline of CD4⁺ T cells associated with rapid onset of an AIDS-like disease[3,29,30]. Four weeks after the final immunization boost, we challenged the groups of mimotope-immunized and wild-type–injected monkeys, together with three naive rhesus macaques, by intravenous injection with 60 monkey infectious doses (MID_{50}) of cell-free SHIV-89.6PD (Fig. 2).

We determined post-challenge viral loads in plasma by a real-time polymerase chain reaction with a detection limit of 200 virus RNA copy equivalents per ml (ref. 31). All of the monkeys became infected; the naive monkeys and those injected with wild-type control phage (mock-immunized) showed detectable viremia as early as 5 days after challenge, with peak viremia at day 12 (Fig. 2*a*). Naive monkeys exhibited peaks of viremia ranging from 1.6–4.5 × 10^6 RNA copies per ml (mean 2.6 × 10^6) at day 12. This was followed by partial control of the acute infection, characterized by viral copy numbers of 0.3–to 1 × 10^5 copies per ml throughout the chronic infection period (Fig. 2*a*). Mock-immunized monkeys experienced high peaks of viremia (range 2.1 × 10^6–20 × 10^6 copies per ml; mean value 9.3 × 10^6 copies per ml) followed by partial clearance of viremia at week 4 and then by a steady increase in the plasma viral copies over the observation time, with final viral titers of 0.5–16.5 × 10^5 copies per ml (Fig. 2*a*). The mimotope-immunized monkeys had variable amounts of plasma viremia (Fig. 2*a*). Monkey 490 had a modest viremia at day 12 after infection: 1 × 10^4 copies per ml, with a delayed peak of 1.3 × 10^5 copies per ml at day 19, substantially lower than the peaks in naive and mock-immunized monkeys. This monkey's viremia cleared rapidly and virus was undetectable at day 50 after infection (<200 viral copies per ml); the virus titer rebounded at day 65 and then spontaneously declined again to undetectable by day 110. Monkey 493 had peak viremia of 0.5 × 10^6 copies per ml at week 2 after infection, followed by a complete clearance of viremia at week 5 and undetectable virus titer for the remainder of the observation period (Fig. 2*a*). In this monkey, no viral copies were detected in inguinal lymph node cells by *in situ* hybridization, and by cocultivation of peripheral blood mononuclear cells (PBMC) with phytohemagglutinin-activated PBMC from uninfected monkeys (Web Fig. C). Monkeys 480, 481 and 485 showed viral peaks within the range of the values observed in naive and wild-type phage–immunized monkeys. Monkeys 481 and 485 showed low or undetectable viremia after primary infection, with virus titers ranging from 1.9 × 10^4 copies per ml to undetectable. In monkey 480, the monkey with the poorest response to immunogen, the peak of viremia was fol-

lowed by a steady increase in plasma viral copies up to 4.9×10^6 per ml at week 22, at which time the monkey was euthanized. Statistical analyses of the viral loads on day 12 showed no significant difference between the two control groups (naive and mock-immunized monkeys) and between the naive or the mock-immunized group and the mimotope-immunized monkeys (P = 0.20, two-tailed Wilcoxon rank sum test). To take into account the variability among monkeys, we carried out statistical analyses of the viral set points by measuring the median of three consecutive plasma viremia levels at days 80, 95 and 110 for each monkey. No significant difference was observed between the control groups (P = 0.057), indicating that the two groups could be pooled together for a statistical analysis. We therefore compared the viral loads in the group of mimotope-immunized monkeys consisting of 481, 485, 490 and 493 (with monkey 480, the single mimotope-immunized monkey that did not mount an antibody response, omitted) to those in the pool of control monkeys. The antibody response elicited by the immunogen correlated significantly with the low plasma viremia at viral set points, in that the viral loads in the mimotope-immunized monkeys were significantly lower than those in the pooled control monkeys (P = 0.012).

Infection with SHIV-89.6P and its derivative SHIV-89.6PD induces a profound decline of peripheral CD4⁺ T lymphocytes[3,30]. Accordingly, both naive and mock-immunized monkeys had a rapid depletion of CD4⁺ T cells at day 12 after infection (Fig. 2*b*), concomitant with the high peaks of plasma viremia (Fig. 2*a*). With the exception of naive monkey 478 and mock-immunized monkey 484, which showed transient detectable titers of CD4⁺ T cells, these monkeys had a profound and irreversible depletion of peripheral CD4⁺ T lymphocytes throughout the observation time (Fig. 2*b*). In the mimotope-immunized group, monkeys 490, 481 and 493 had a significant decrease in their CD4⁺ T cells during the acute phase of viremia, followed by a partial recovery to 250–500 CD4⁺ T cells per μl (40–60% of the prechallenge titers; Fig. 2*b*). Similar titers of peripheral CD4⁺ T cells are associated with absence of AIDS-like illness in HIV-1–infected subjects[32,33]. Monkey 485 showed a prolonged decline of peripheral CD4⁺ T lymphocytes through week ten, followed by increasing numbers of CD4⁺ T cells that resulted in final CD4⁺ T-cell titers similar to those observed in monkeys 490, 481 and 493. Monkey 480 had an irreversible loss of peripheral CD4⁺ T lymphocytes to less than 1% of the initial titer (Fig. 2*b*). We carried out statistical analyses of the peripheral CD4⁺ T cell counts after acute infection as described in the case of viral loads, by analyzing the median values of three consecutive CD4⁺ T cell-determinations. The CD4⁺ T cell-counts of the group of mimotope-immunized monkeys (monkeys 481, 484, 490 and 493) were significantly higher than those of the pooled control monkeys (P = 0.0061).

Immune response after challenge

Because of the profound depletion of CD4⁺ T cells, few or no HIV-specific antibodies are detected in unprotected SHIV-89.6PD–infected monkeys[3,30]. Consistent with this pattern, we found that two naive monkeys (WAT and XKH) and three mock-immunized monkeys (483, 492 and 497) did not show a detectable antibody response (Fig. 3*a*). Naive monkey 478 and mock-immunized monkey 484 showed an envelope-specific antibody response at week seven after challenge and declining titers thereafter (Fig. 3*a*). In contrast, the mimotope-immunized monkeys 481, 485, 490 and 493 showed a rapid increase from prechallenge levels of antibody to oligomeric gp140-89.6, with titers rising as high as 2.5×10^5 (Fig. 3*a*). One monkey (480) showed a dramatic decline in CD4⁺ T cells and did not show a detectable Ab response to the challenge virus (Fig. 3*a*).

We analyzed neutralizing antibodies in cultures of monkey PBMCs infected with a stock of SHIV-89.6PD expanded in PBMCs from rhesus macaques. Antibodies from prechallenge sera of mimotope-immunized monkeys showed neutralization titers of 1:10 in monkeys 481 and 485 and of 1: 30 in monkeys 490 and 493; these mimotope-immunized monkeys manifested increased titers of neutralizing antibodies against the challenge virus starting at week four after challenge (Fig. 3*b*). No neutralizing activity was detected in monkey 480 (Fig. 3*b*). Within the control groups, low titers of neutralizing antibody were transiently detected in naive monkey 478 and mock-immunized monkey 484 (Fig. 3*b*). These results indicate that monkeys primed by mimotope immu-

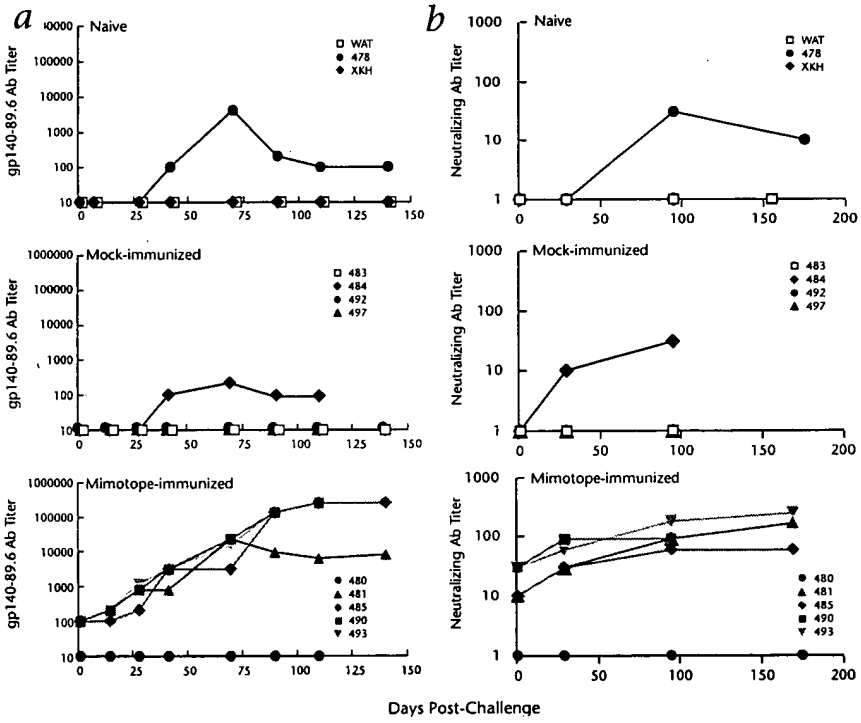

Fig. 3 Post-challenge antibody response. *a*, Antibody titers were determined at the indicated time points by ELISA. Linear dilutions of monkeys' sera were tested for binding to a preparation of oligomeric gp140-89.6 as reported[42]. *b*, Analysis of neutralizing antibody response. Virus neutralizing antibodies were determined by using rhesus macaque PBMCs infected with SHIV-89.6PD (see Methods). Neutralization titers were defined as the plasma dilution that resulted in >90% reduction of viral production.

Table 1 Amino acid sequences of the HIV-1 epitopes

Epitope[a]	Amino acid sequence	Assignment to regions of gp160
p195	KSSGKLISL	gp120 V1[b]
p217	CNGRLYCGP	gp120 C2[c]
p197	GTKLVCFAA	gp41[d]
p287	CAGGLTCSV	Undetermined[e]
p335	SGRLYCHESW	Undetermined

[a], Epitopes were selected by screening random peptide libraries as reported[17]. [b], Epitope p195 shares sequence homology with the gp120 V1 region (residues 112–120) of HIV-1–U16374, a primary isolate from an acute HIV seroconverter[22]. [c], p217 sequence matches the C2 region (residues 198–205) of HIV-1–U11607, a primary isolate from an AIDS patient[23]. [d], Epitope 197 maps to a region of gp41 (residues 599–608) of the primary HIV-1 isolate HIVANT70 (ref. 26). [e], Although no obvious sequence homology with HIV proteins was detected in the case of p287 and p335, previous studies have indicated that these epitope are antigenic mimics of gp160 epitopes[17].

nization have a rapid and durable antibody response to the challenge virus.

As phage-displayed peptides have been reported to stimulate specific T cell responses[21], we analyzed PBMC obtained from the mimotope-immunized monkeys on the day of viral challenge for the presence of T cells capable of producing intracellular IFN-γ upon stimulation with peptide mimotopes, a sensitive assay of epitope-specific immune responses[34]. In these experiments, no intracellular IFN-γ production was induced by pooled peptide mimotopes, indicating that the selected peptides do not function as T cell epitopes (Web Table A).

Clinical events associated with viral challenge

Naive and mock-immunized monkeys experienced severe illness starting at week six after challenge. This included untreatable anemia, fever, multiple infections, hemorrhagic diarrhea and weight loss; the degree of illness required euthanasia. Among the mimotope-immunized monkeys, monkey 480, which showed no immune response to the immunogen before challenge and high viremia and dramatic loss of $CD4^+$ T cells after challenge, developed an AIDS-like illness similar to that observed in naive and wild-type phage–immunized monkeys. Monkey 490 developed acute glomerulonephritis followed by anuria and heart failure, along with low viremia, conserved $CD4^+$ T cell titer and a robust antibody response to SHIV-89.6PD. Histological analysis of tissues revealed a focal necrosis of kidney tubules and glomeruli, with massive deposits of proteins; lymph nodes showed numerous macrophages and prominent erythrophagocytosis. This histopathologic pattern is not characteristic of disease resulting from SHIV-89.6PD infection and resembles the immune-complex disease associated with aberrant immune responses[35]. In addition, monkey 490 experienced a focal subendocardial myocyte necrosis with neutrophilic inflammation that might have contributed to the heart failure. No abnormalities were detected in other tissues, including liver, lymph nodes, spleen and lung (Web Figs. D,E). These findings indicate that the cause of death in monkey 490 was probably unrelated to poor control of SHIV-89.6PD infection. The remaining three mimotope-immunized monkeys (481, 485 and 493) have remained healthy, with no reported clinical events for a period of 270 days at the time of this writing.

Discussion

To cover the high antigenic polymorphism of HIV-1 isolates, an effective HIV-1 vaccine should include immunogenic epitopes shared by different clades and quasispecies from distant geographic regions. Vaccine formulations that deliver envelope proteins from a single virus strain, or a pool of envelopes, have failed to induce a significant degree of protection against challenge with heterologous viruses[11,36] and might well afford little or no protection against field isolates[12,13]. To address this difficulty, we isolated a pool of phage-displayed epitopes from RPLs by taking advantage of the HIV-specific antibody repertoire induced by natural infection[17]. The selected phage-borne peptides are antigenic mimics of conformational epitopes of gp120 and gp41 generated *in vivo* in the course of natural infection[17] and are shared by a substantial percentage of HIV-1 strains of non-B clades, including clades A, C, D, E, and F (Table 1). These findings indicate that the selected epitopes might induce broad antibody responses in primates without the constraint of a predefined envelope with narrow specificity. In support of this concept, we have shown that bacteriophage displaying epitope determinants of HIV-1 gp160 are immunogenic in nonhuman primates and are endowed with protective properties. In this regard, four of five mimotope-immunized rhesus monkeys raised anti-envelope antibody responses during the immunization followed by a rapid increase in antibody titers after challenge with SHIV-89.6PD. In contrast to control monkeys, these monkeys showed reduced peaks of plasma viremia, with low or undetectable viremia set points during the chronic phase of infection. They retained substantial titers of $CD4^+$ T lymphocytes and remained free of illness related to SHIV-89.6PD infection. These findings are consistent with human studies, in which low viral loads are associated with a favorable prognosis[37]. In addition, low or undetectable viremia in the absence of AIDS-like illness might result in reduced rates of viral transmission[38,39] and could be advantageous in populations affected by high rates of HIV-1 infection.

The results of this study are comparable with those recently obtained in monkeys using a combination of DNA vaccination with administration of an IL-2 plasmid[40] or DNA priming followed by recombinant modified vaccinia Ankara (rMVA) booster[41]. In these studies, protection against disease progression was correlated with cell-mediated immune responses against SHIV. Here we have not detected a contribution of SHIV-specific cell-mediated immune responses in the observed effects of immunization. Indeed, the observed protection was probably medi-

Table 2 ELISA reactivities of HIV-1 epitopes with HIV-positive sera

	HIV-infected subjects		
Clade[a]	Number of tested samples[b]	Number of positive samples	% of positive samples
B	120	119	99.1
A	25	22	92.0
D	26	25	96.1
C	22	20	90.1
E	19	13	68.4
F	8	6	75.0

[a], Serum samples from USA, Uganda, Nigeria, India, Thailand and Peru were assigned to specific clades as reported[44]. [b], ELISA reactivities of HIV-1–specific epitopes were detected as reported[17]. Serum antibodies were tested for binding to each of the phage-displayed epitopes; values were considered positive when at least 4-fold ± 2 s.e.m. higher than the background signal of wild-type phage was detected. A detailed representation of ELISA reactivities is shown in Web Fig. F.

Table 3 ELISA reactivities of HIV-1 epitopes with SHIV-positive sera

	SHIV-infected monkeys	
Virus strain[a]	Number of tested monkeys[b]	Number of positive monkeys
SHIV-DH12	17	17
SHIV-89.6	9	9
SHIV-89.6PD	21	19
SHIV-W6.1D	4	4
SHIV-Han2	2	2
SIVmac239	12	0

[a], Rhesus macaques were infected with the indicated SHIV chimeric strains carrying envelopes from primary HIV-1 isolates as reported[3]. [b], antibodies from infected monkeys were tested at different times after infection for reactivity with a pool of ten HIV-1 specific epitopes displayed on phage as described[17]. Values were considered positive when at least 4-fold ± 2 s.e.m. higher than the background signal of wild-type phages. No ELISA reactivity was detected in the case of antibodies from SIV-infected monkeys. A detailed representation of ELISA reactivities is shown in Web Fig. G.

ated by the antibody responses to SHIV-89.6PD envelope induced by the immunogen. In fact, the favorable outcome was restricted to the group of four monkeys (481, 485, 490,. 493) that manifested a prechallenge antibody response to oligomeric gp140-89.6 envelope followed by a rapid increase in the antibody response (Fig. 3*a*). Consistently, antibodies from these monkeys effectively neutralized the challenge SHIV-89.6PD virus in an *in vitro* infection assay (Fig. 3*b*). The protection observed was achieved upon infection with the highly virulent SHIV-89.6PD strain, raising the possibility that the degree of protection afforded by mimotope immunization might be greater with a less virulent challenge. Notably, monkeys were immunized with conformational epitopes shared by a substantial number of virus isolates of different subtypes. Thus, immunization with HIV-1 mimotopes overcomes the constraint of vaccination regimens that rely on predetermined virus strains, and might afford protection from infection with heterogeneous field isolates.

Methods

Vaccine trial. Rhesus macaques were maintained in accordance with the *American Association for Accreditation of Laboratory Animal Care Standards* and housed in a biosafety level 2 facility. The envelope-specific epitopes used as immunogen were isolated from RPLs displayed on filamentous f.11.1 as reported[17]. A pool of five epitopes (p195, p197, p217, p287 and p335) was injected into each quadricep of juvenile rhesus macaques 480, 481, 485, 490 and 493 at 4×10^{13} physical particles each with 100 μg of QS21 adjuvant (Aquila Biopharmaceuticals, Framingham, MA). In parallel, rhesus macaques 483, 484, 492 and 497 were immunized intramuscularly with 2×10^{14} physical particles of f.11.1 wild-type phage with 100 μg of QS21 adjuvant. Monkeys were matched for gender and body weight and inoculated five times at ten-week intervals. Viral challenge was performed at week 44 by intravenous inoculation of 60 MID_{50} (as previously determined) of a SHIV-89.6PD preparation (3,500 $TCID_{50}$ per ml) expanded on monkey PBMCs as reported[3]. After challenge, monkeys were monitored for plasma viral load by detecting SHIV RNA using a real-time PCR method[31], with minor modifications. Briefly, 500 μl of plasma was added to 1 ml of Dulbecco PBS and spun for 1 h at 10,000*g*. The viral pellet was lysed using RNASTAT-60 (Tel-Test 'B', Friendwood,TX) according to the manufacturer's directions. The samples were then amplified by using the following primers and probe: SIV-F, 5'-AGTATGGGCAGCAAATGAAT-3'; SIV-R, 5'-TTCTCTTCTGCGTGAATGC-3'; SIV-P, 5'-6FAM-AGATTTGGATTAGCAGAAAGCCTGTTGGA-TAMRA-3'. If plasma viremia was not detected, PBMCs from infected monkeys (1×10^6 per ml) were stimulated with 10 μg/ml PHA (Sigma-Aldrich) for two days and cocultured with equal titers of PHA-activated PBMCs from uninfected rhesus macaques. Reverse transcriptase activity and p27 production were determined every three days over a three-week infection time. Titers of peripheral lymphocytes positive for CD2, CD3, CD4, CD8, CD29 and CD20 were determined by flow cytometry by using the following mAbs: CD2-FITC, CD4-PE, CD8-PE, CD20-PE (Becton Dickinson, San Jose, California), CD3-FITC and CD29-PE (Pharmingen, San Diego, California). Monkeys were monitored clinically, by routine hematological testing and by blood-chemistry measurements. Monkeys were then subjected to necroscopy followed by histological analysis of tissues, including brain, kidney, large and small intestine, lung, liver, lymph nodes, myocardium and spleen.

Antibody detection. Titers of antibodies to peptide mimotopes and to oligomeric envelope proteins were determined by ELISA as described[17,42]. In brief, Immunolon-2 (Dynex Technology, Chantilly, VA) 96-well U-bottom plates were coated overnight with 5 μg/ml of either peptide mimotopes or oligomeric gp140 (89.6 strain). Twofold serial dilutions of monkey plasma were incubated overnight at 37 °C in blocking buffer containing 5% goat serum and 0.02% (w/v) sodium azide. After washing, alkaline phosphatase–conjugated anti-monkey Ab (Fc-specific) was added for 1 h and the plates were developed. Results were expressed as the difference between OD_{405} and OD_{620}. Assays were performed in duplicate. The following peptides were used: p195, EGEFCKSSGKLISLCGDPA; p197, EGEFCQTKLVCFAAAGDPA; P217, EGEFCCNGRLYCQPCGDPA; p287, EGEFCCAGQLTCSVCGDPA; p335, EGEFCSGRLYCHESWCGDPA. Antibody reactivities to V3 loop were detected by ELISA using the peptides RPNTRERLSIGPGRAFYA (89.6P V3) and KSIHIGPGRAFYTTG (V3 consensus subtype B) together with scrambled control peptides.

The neutralizing properties of monkey plasma were assayed using monkey PBMC as reported[43]. The virus stock consisted of the SHIV-89.6PD inoculum used for the monkey's challenge and expanded on PHA-activated PBMCs from naïve rhesus macaques. Triplicate plasma dilutions were incubated for 1.5 h at 37 °C with 20 μl of previously titrated virus aliquots (1.1×10^5 reverse transcriptase-cpm per 15 ng of p27 per 100 $TCDI_{50}$) in a total volume of 100 μl. Preimmunization plasma was used as negative control. The mixtures were added to rhesus macaque PBMCs (4×10^5 per 200-μl culture) previously activated with PHA (10 μg/ml) for 36 h. After a 24-h incubation, medium was removed by centrifugation and replaced. Cultures were monitored every three days by reverse transcriptase assay. End-point titrations were calculated at day seven as the plasma dilution that resulted in > 90% virus inhibition.

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Evidence for Similar Recognition of the Conserved Neutralization Epitopes of Human Immunodeficiency Virus Type 1 Envelope gp120 in Humans and Macaques

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We compared the immune responses to the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins in humans and macaques with the use of clade A and clade B isogenic V3 loop glycan-possessing and -deficient viruses. We found that the presence or absence of the V3 loop glycan affects to similar extents immune recognition by a panel of anti-HIV human and anti-simian/human immunodeficiency virus (anti-SHIV) macaque sera. All sera tested neutralized the glycan-deficient viruses, in which the conserved CD4BS and CD4i epitopes are more exposed, better than the glycan-containing viruses. The titer of broadly neutralizing antibodies appears to be higher in the sera of macaques infected with glycan-deficient viruses. Collectively, our data add legitimacy to the use of SHIV-macaque models for testing the efficacy of HIV-1 Env-based immunogens. Furthermore, they suggest that antibodies to the CD4BS and CD4i sites of gp120 are prevalent in human and macaque sera and that the use of immunogens in which these conserved neutralizing epitopes are more exposed is likely to increase their immunogenicity.

The development of an effective and safe AIDS vaccine would greatly benefit from the use of nonhuman primate models to assess and compare candidate immunogens for their protective potential. In this regard, the simian immunodeficiency virus (SIV)-macaque system has served as a valuable model for the evaluation of various vaccine strategies and antiviral therapies against AIDS (59). Nevertheless, due to the genetic, antigenic, and immunogenic differences between the SIV and human immunodeficiency virus (HIV) envelopes, the SIV system cannot be used to address directly the efficacy of HIV type 1 (HIV-1) Env-based immunogens. Towards this end, chimeric simian/human immunodeficiency viruses (SHIVs) that carry the *tat*, *rev*, and *env* genes of HIV-1 on the genomic backbone of the pathogenic SIVmac239 strain have been constructed (28, 31, 46). Through serial passage or in vivo adaptation, several pathogenic SHIVs have been obtained and characterized (17, 18, 20, 22, 29, 30, 46, 53). These viruses cause disease in macaques when inoculated by intravenous and mucosal routes, thus providing a system whereby the ability of HIV-1-based immunogens to protect against infection, reduce viral load, or delay progression to disease can be assessed. The relative efficacies of different vaccine designs and concepts can also be tested. Indeed, there is an increasing use of the SHIV-macaque model to evaluate different HIV-1 Env-based experimental vaccines such as envelope subunits, DNA vaccines, and various antibodies for passive immunization (3, 4, 33, 34, 51, 52, 59). However, the central question of the extent to which information obtained in the SHIV-macaque model, particularly with regard to humoral immune protection, can be extrapolated or applied to the human setting remains unclear. Limited data are available that directly compare the antigenicity (that is, ability to bind antibodies) and immunogenicity (that is, ability to elicit antibodies) of the HIV-1 envelope in humans versus nonhuman primates.

There is mounting evidence to suggest that both antibody and cellular immune responses will be required to effectively control HIV infection and spread. Antibody responses to the HIV-1 envelope glycoproteins during natural infection have been widely investigated (6, 44). These studies revealed several neutralization targets on envelope gp120, among which are the CD4 binding site (CD4BS) (7, 23, 45); the variable V1, V2, and V3 loops; a gp120 structure that is near the chemokine receptor binding site and which is better exposed following CD4 binding (CD4i epitopes) (27, 49, 50, 55, 61); and the unique 2G12 epitope (56). Antibodies to the V2 and V3 regions are mainly isolate or type specific, whereas those reacting with the discontinuous CD4BS and CD4i epitopes are broadly neutralizing (44). Longitudinal studies showed that strain-specific antibodies arise relatively early in infection (2, 24, 38, 57), while the broadly neutralizing antibodies develop later in infection (1, 8, 35, 36, 60). Although neutralization of viruses adapted to growth in T-cell lines (TCLA viruses) can be easily achieved with sera obtained from infected individuals, primary isolates are much more resistant (10, 40). The low frequency and titers with which broadly neutralizing antibodies are detected in HIV-1-infected individuals has led to the suggestion that the conserved neutralization epitopes of gp120, such as the CD4BS and CD4i sites, are poorly immunogenic.

The generation and specificity of neutralizing antibody responses to HIV-1 envelopes in monkeys infected with TCLA HIV-1-derived ($SHIV_{HXB2}$ and $SHIV_{KU}$) or dual-tropic primary-isolate-derived ($SHIV_{89.6}$ and $SHIV_{89.6PD}$) (nonpathogenic and pathogenic, respectively) SHIVs have also been evaluated. Strong neutralizing antibody responses against homologous viruses that are directed against the V1-V2 and V3 epitopes (15, 37) can be readily detected early in infection. Similar to the case for infections in humans, however, titers of

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TABLE 1. Viral envelope gp120 glycoproteins used

gp120	V3 glycan[a]	Coreceptor use[b]	Clade
SF33 WT	−	X4	B
SF33 V3T	+	X4	B
SF162 WT	+	R5	B
SF162 V3A	−	R5	B
SF170 WT	+	R5	A
SF170 V3A	−	R5	A

[a] + and −, presence and absence, respectively, of the N-linked glycan at amino acid 301 of envelope gp120 (numbered according to the prototype HXBc2 sequence [25]).
[b] X4, CXCR4 using; R5, CCR5 using.

neutralizing antibodies to heterologous SHIVs or primary HIV-1 isolates are generally low or undetectable and require a longer infection time to develop. In accordance, protective immunity has been demonstrated in homologous $SHIV_{IIIB}$ challenge experiments, while heterologous challenge with viruses expressing divergent envelope glycoproteins ($SHIV_{89.6}$) still resulted in infection (51). Thus, the conserved neutralization domains of gp120 also appear to be poorly immunogenic in macaques.

With the hope of inducing protective immune responses, focus has now been directed towards designing Env-based vaccine immunogens in which the conserved functional domains of gp120 are better exposed. We recently showed that the presence of a highly conserved N-linked glycosylation site located at the N terminus of the V3 loop modifies the structure of the V3 loop and obstructs access to the highly conserved CD4 binding and CD4i sites of gp120 from diverse HIV-1 isolates (32). The use of V3 loop deglycosylated Envs as vaccine components may therefore elicit broadly cross-reactive neutralizing activity. Moreover, by assessing the degree to which the V3 loop glycan affects susceptibility of the virus to neutralization by sera from infected humans and macaques, one may gain insights into the similarities or differences in antigenic recognition of the HIV-1 envelopes by humans and macaques. Furthermore, the extent to which the conserved neutralization epitopes of gp120 are immunogenic in the two hosts could be evaluated. In the present study, the antibody responses to the HIV-1 envelope in human and nonhuman primates were compared using V3 loop glycan variants and a panel of sera collected from HIV-1-infected individuals and SHIV-infected rhesus macaques. The SHIVs carry the envelope gene of the T-cell-line-tropic, CXCR4-using (X4), and V3 loop glycan-deficient strain $HIV\text{-}1_{SF33}$ or the macrophage-tropic, CCR5-using (R5), and V3 glycan-possessing isolate $HIV\text{-}1_{SF162}$ ($SHIV_{SF33}$ and $SHIV_{SF162}$, respectively) (31). Thus, an opportunity is also provided to examine whether immunization with glycan-deficient envelopes that better expose highly conserved epitopes will elicit more potent broadly cross-neutralization antibodies.

MATERIALS AND METHODS

Cells. Human osteosarcoma (HOS) cells engineered to express CD4 (T4) and the chemokine receptors CXCR4 and CCR5 (HOS T4 pBabe, HOS T4 X4, and HOS T4 R5 cells, respectively) were obtained from N. Landau (Salk Institute, La Jolla, Calif.). The cells were maintained in Dulbecco's modified Eagle's medium containing 1 μg of puromycin per ml, 10% fetal bovine serum (FBS), and antibiotics. 293-T cells were cultured in Dulbecco's modified Eagle's medium without puromycin.

Viruses and sera. The construction of envelope (Env) expression vectors and the generation of single-round replication-competent luciferase reporter viruses have been previously described (11, 32). Briefly, the coding fragments of wild-type (WT) and V3 glycosylation mutant Envs were subcloned into the mammalian expression vector pCAGGS (11). The Env expression plasmids together with the HIV-1 NL-Luc-E^-R^- vector (14) were then cotransfected by lipofection (DMRIE-C Reagent; Gibco BRL, Gaithersburg, Md.) into 293-T cells. Cell culture supernatants were harvested at 72 h posttransfection, filtered through 0.45-μm-pore-size filters, and stored at −70°C. Viruses were quantitated by a $p24^{gag}$ enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, North Chicago, Ill.). The biologic characteristics of the viruses are listed in Table 1. The sources and gp120 ELISA titers of SHIV and HIV polyclonal sera used are listed in Table 2. The clade B human polyclonal antisera were collected from HIV-1-infected long-term nonprogressors residing in the United States. The 2743 M clade A serum is from an HIV-1-positive patient in Rwanda and was a kind gift of Linqi Zhang (Aaron Diamond AIDS Research Center, New York, N.Y.).

gp120 ELISA. To measure viral envelope glycoprotein-specific antibody endpoint titers of serum samples from SHIV-infected macaques or HIV-infected individuals, 96-well Immulon plates were coated with recombinant $HIV\text{-}1_{SF2}$ gp120 (kindly provided by Chiron Corp., Emeryville, Calif.). After blocking of the coated wells with 4% (wt/vol) nonfat dry milk and 10% FBS diluted in Tris-buffered saline (TBS) (25 mM Tris base, 144 mM NaCl, pH 7.5), serially diluted human or monkey serum (in TBS–FBS–milk–1% NP-40) was added to each well and incubated for 2 h at room temperature. The wells were then extensively washed with TBS and incubated with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) for 2 h at room temperature. The conjugate was then removed by extensive washing, the well was incubated with Dako AMPAK substrate-amplifier (Zymed Laboratories Inc., South San Francisco, Calif.) to achieve color development, and the reaction was stopped by the addition of 0.4 N sulfuric acid. Antibody reactivity to gp120 was then determined by measuring the optical density (OD) at 485 nm, using an automated plate reader. Endpoint titers are reported as the last serial dilution whose OD was three times that of normal monkey or normal human serum or as an OD of 0.1, whichever value was greater. All endpoint titers represent at least two independent experiments.

Neutralization assay. Neutralization was performed using HOS T4 pBabe, HOS T4 X4, and HOS T4 R5 cells in 96-well plates. Briefly, cells were plated and pretreated with Polybrene (2 μg/ml; Sigma, St. Louis, Mo.). A 0.5- to 1.0-ng $p24^{gag}$ equivalent amount of each pseudotyped reporter virus was preincubated, in duplicate, with serial dilutions of sera for 1 h at 37°C and then added to cells. The infected cells were cultured for 72 h at 37°C before being lysed and tested for luciferase activity according to the instructions of the manufacturer (Promega, Madison, Wis.). Luciferase activity associated with the cell lysate was detected with an MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, Va.). Infection of coreceptor-bearing cells with NL4-3 virus generated

TABLE 2. Sera used

Serum	Source[a]	Time of collection (wpi)	Anti-gp120 ELISA end point titer
M25814	$SHIV_{SF33}$	4	1:700
		8	1:4,000
		24	1:5,500
		32	1:20,000
		53	1:1,000
		96	1:20,000
M26131	$SHIV_{SF33}$	53	1:320
M26240	$SHIV_{SF33}$	53	1:780
E239	$SHIV_{SF33A.2}$	53	1:1,050
M565	$SHIV_{SF33A.5}$	53	1:1,050
T528	$SHIV_{SF162}$	57	1:1,100
T373	$SHIV_{SF162}$	79	1:560
M26419	$SHIV_{SF162}$	52	1:560
GJ	Polyclonal human clade B serum		1:110,000
GS0	Polyclonal human clade B serum		1:70,000
GS21	Polyclonal human clade B serum		1:70,000
2743 M	Polyclonal human clade A serum		1:35,000

[a] For SHIV sera, the virus strain is indicated; infection was by the intravenous route.

in the absence of Env or infection of HOS T4 pBabe cells lacking coreceptor served as negative controls.

RESULTS

V3 loop glycan modulates the antigenicity of X4 and R5 gp120 envelope. We previously reported that the lack of an N-linked glycosylation site at the N terminus of the V3 loop of $SHIV_{SF33}$ (amino acid 301, numbered according to the prototype HXBc2 sequence) (25) was partially responsible for sensitivity of the virus to neutralization with a mixture of anti-HIV-1 sera (9). The presence of serum antibodies directed at the site that is exposed or modulated by the absence of the V3 loop glycan from one infected individual within the mixture, however, would have resulted in the pattern of neutralization observed. To determine the prevalence of antibodies that are directed against this cryptic epitope in infected individuals and to assess the degree to which the V3 loop glycan affects antibody recognition, the susceptibilities of reporter viruses pseudotyped with WT HIV-1_{SF33} and V3 mutant Env to a panel of HIV-1-positive sera were determined. The WT virus lacks the V3 loop glycan, whereas the V3 mutant virus contains the glycan moiety. To examine whether this V3 loop glycan also modulates immune recognition of primary HIV-1 viruses by sera from infected individuals, isogenic V3 loop glycan-possessing (WT) and -deficient (mutant) reporter viruses of clade B HIV-1_{SF162} and clade A HIV-1_{SF170} (Table 1) were also analyzed. The results are shown in Fig. 1.

We found that the absence or presence of the V3 loop glycan on the TCLA, X4 virus HIV-1_{SF33} or the macrophage-tropic, R5 strain HIV-1_{SF162} altered their sensitivity to neutralization with the three HIV-1 clade B antisera tested (Fig. 1A). Consistently, the V3 glycan-deficient HIV-1_{SF33} WT and HIV-1_{SF162} V3A viruses were more sensitive to neutralization than their glycan-possessing counterparts. Whereas a two- to threefold difference in 90% inhibitory concentration (IC_{90}) was observed for the HIV-1_{SF33} WT and the glycan-containing V3T mutant viruses, a 10- to 20-fold increase in neutralization sensitivity was seen for the glycan-deficient HIV-1_{SF162} V3A virus compared to the parental virus. In contrast, the HIV-1_{SF170} WT and V3 glycan-lacking viruses were relatively resistant to neutralization with the sera tested. Only serum GS21 achieved 90% neutralization at a 1:20 dilution, with no difference in titer for the glycan-possessing or -deficient viruses. Interestingly, the glycan-possessing R5 primary clade B HIV-1_{SF162} appeared to be more sensitive to neutralization than the glycan-possessing TCLA, X4 variant HIV-1_{SF33} V3T. Perhaps this reflects a similarity in the gp120 antigenic structures of primary viruses that establish infection in vivo.

To examine whether genotypic variation underscores the inability of clade B sera to neutralize the HIV-1_{SF170} viruses, neutralization with a clade A anti-HIV-1 antiserum (2743 M) was performed (Fig. 1B). A pattern similar to that of neutralization with clade B anti-HIV antisera was observed, with the V3 glycan-deficient HIV-1_{SF33} WT and HIV-1_{SF162} V3A mutant viruses being more susceptible to neutralization with the 2743 M serum than their corresponding V3 glycan-containing viruses. Again, a greater difference between the neutralization susceptibilities of the clade B primary HIV-1_{SF162} WT and V3A mutant viruses compared to the HIV-1_{SF33} glycan-possessing and -lacking viruses was observed, with the V3 loop glycan-deficient HIV-1_{SF162} V3A virus being significantly more sensitive (IC_{90}, ≈1:10,000). Weak neutralization of the clade A V3 glycan-deficient HIV-1_{SF170} V3A virus (IC_{50}, 1:80) was achieved, but the glycan-possessing WT virus was resistant. Thus, the antienvelope response in this particular clade A serum appears to be similar to that of the clade B sera.

Immune responses to HIV-1 gp120 are comparable in humans and macaques. The findings with the human antisera indicated that the presence or absence of V3 loop glycan influenced the antigenicity of envelope gp120. To assess whether macaques recognize the HIV-1 gp120 Env to the same extent as humans, the ability of sera from $SHIV_{SF33}$- and $SHIV_{SF162}$-infected animals to neutralize the corresponding WT and V3 glycan mutant viruses was examined. The results are presented in Fig. 2 and 3. High titers of homologous neutralization antibodies were observed, again with the presence or absence of the V3 loop glycan modulating the degree of neutralization sensitivity of the viruses. For HIV_{SF33}, the V3 glycan-lacking WT virus is two- to threefold more susceptible to neutralization than the V3 glycan-possessing V3T virus (Fig. 2). Sera from macaque M25814 exhibited the highest anti-gp120 ELISA titer (Table 2) and the greatest neutralization titer. Since the WT virus is the autologous virus for $SHIV_{SF33}$ sera and the V3 loop glycan has been shown to modulate the structure of the V3 loop of HIV-1_{SF33} (32), the modest difference in neutralization susceptibility between the WT and V3T viruses could be attributed to the presence of neutralizing antibodies directed against the V3 loop. However, the data generated with the $SHIV_{SF162}$ sera suggest otherwise. For these sera, the autologous glycan-containing WT virus was found to be two- to threefold more resistant to neutralization than the V3 glycan-deficient V3A mutant virus (Fig. 3). Thus, antibodies other than those directed against the V3 loop are involved in mediating virus neutralization. Collectively, these findings indicate that the immune responses to Env gp120 in macaques are similar to those in humans regardless of whether the immunizing virus is glycan containing or glycan deficient.

Conserved neutralization epitopes of HIV-1 gp120 are immunogenic in humans and macaques. We recently showed that the V3 loop glycan served to block access to major conserved neutralizing epitopes, namely, the CD4BS and CD4i sites, of gp120 of both HIV-1_{SF33} and HIV-1_{SF162}, as well as to CD4BS of HIV-1_{SF170} (32). Viruses that lack the V3 loop glycan are more susceptible to neutralization by anti-CD4BS and anti-CD4i monoclonal antibodies (summarized in Table 3). Antibodies, in particular those directed against CD4BS, are broadly cross-neutralizing. The similar increase in susceptibilities to neutralization of the V3 glycan-deficient HIV-1_{SF33} WT and HIV-1_{SF162} V3A mutant viruses, compared to their V3 glycan-possessing counterparts, by the four heterologous human anti-HIV sera (GJ, GSO, GS21, and 2743 M) (Table 2) raises the possibility that the conserved CD4BS and CD4i epitopes are immunogenic in infected humans. To determine whether these conserved epitopes are also immunogenic in macaques, the ability of $SHIV_{SF33}$ and $SHIV_{SF162}$ sera to neutralize heterologous viruses that contain or lack the V3 loop glycan was determined (Fig. 2 and 3). Since broadly cross-reactive antibodies typically arise later in the course of natural infection (5, 38, 43), sera from macaques that have been infected for over 1

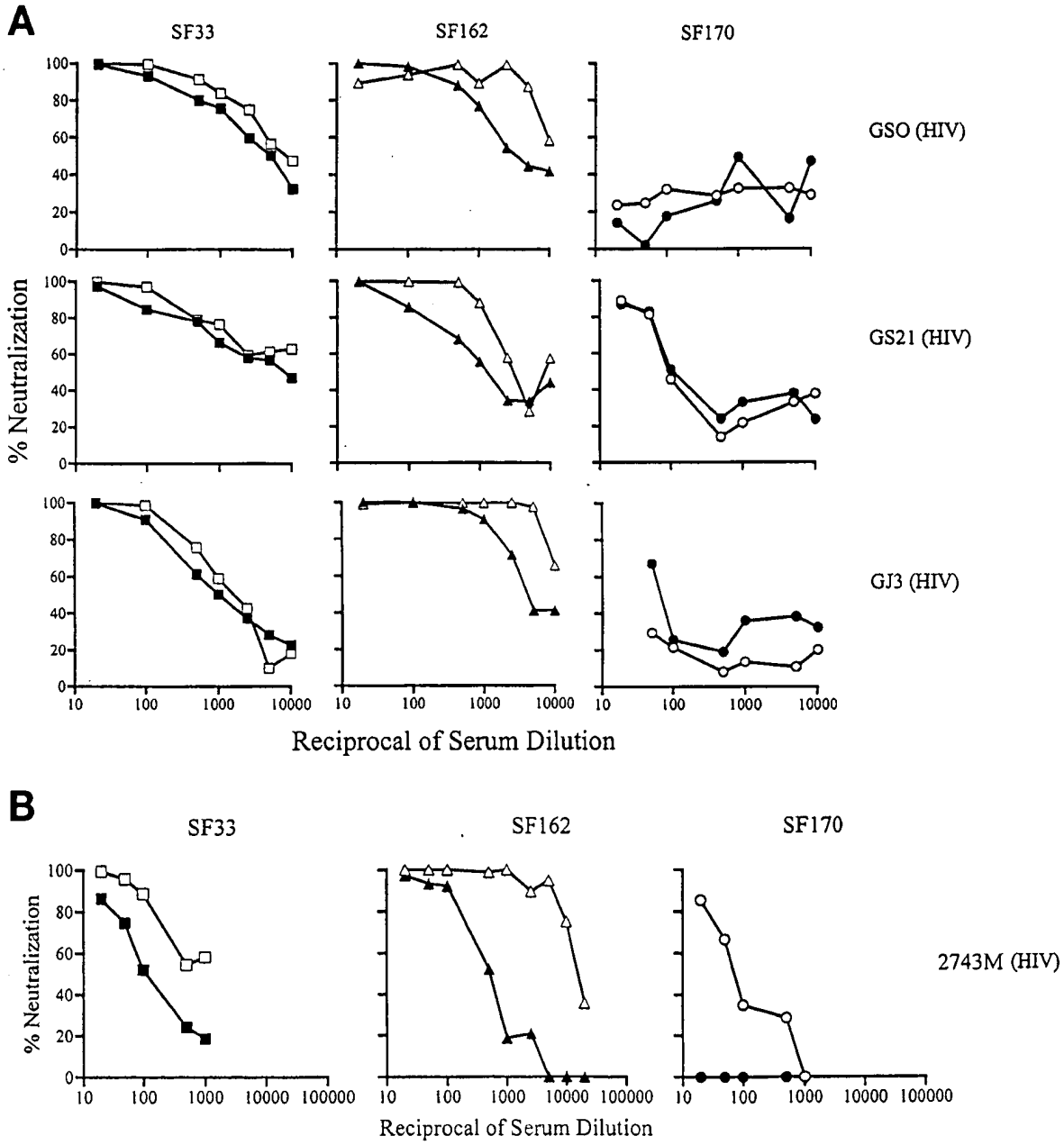

FIG. 1. Neutralization of glycan-possessing and -deficient viruses by human polyclonal anti-HIV-1 sera. (A) Sera from three clade B-infected individuals. (B) Sera from a clade A-infected individual. Open and closed symbols indicate the absence and presence, respectively, of the V3 loop glycan of SF33 (squares), SF162 (triangles), and SF170 (circles) viruses. The data shown are representative of those from at least three independent experiments.

year were used. We found that the $SHIV_{SF33}$ sera exhibited the broadest cross-neutralization (Fig. 2). All three $SHIV_{SF33}$ sera tested achieved 90% neutralization of heterologous isolates, with the degree of cross-neutralization of each $SHIV_{SF33}$ serum correlating with its anti-gp120 ELISA titer. Serum from macaque M25814 had the highest anti-gp120 ELISA titer (1:20,000) (Table 2) and the strongest cross-neutralization potential. An IC_{90} of 1:2,000 against the V3 glycan-deficient HIV_{SF162} V3A and an IC_{90} of 1:40 against the HIV-1_{SF162} WT was observed (Fig. 2). More importantly, this serum also neutralized the clade A HIV-1_{SF170} WT and its corresponding V3A virus at an IC_{90} of 1:50. Sera M26131 and M26240, which exhibited anti-gp120 ELISA titers of 1:320 and 1:780, respectively, cross-neutralized HIV-1_{SF162} V3A at 1:200 and the WT virus at 1:40. These sera also weakly neutralized the clade A HIV-1_{SF170} Env-based viruses (IC_{90} of 1:20 to 1:50). In contrast, the $SHIV_{SF162}$ sera tested did not display significant cross-neutralizing titers even though their anti-gp120 ELISA

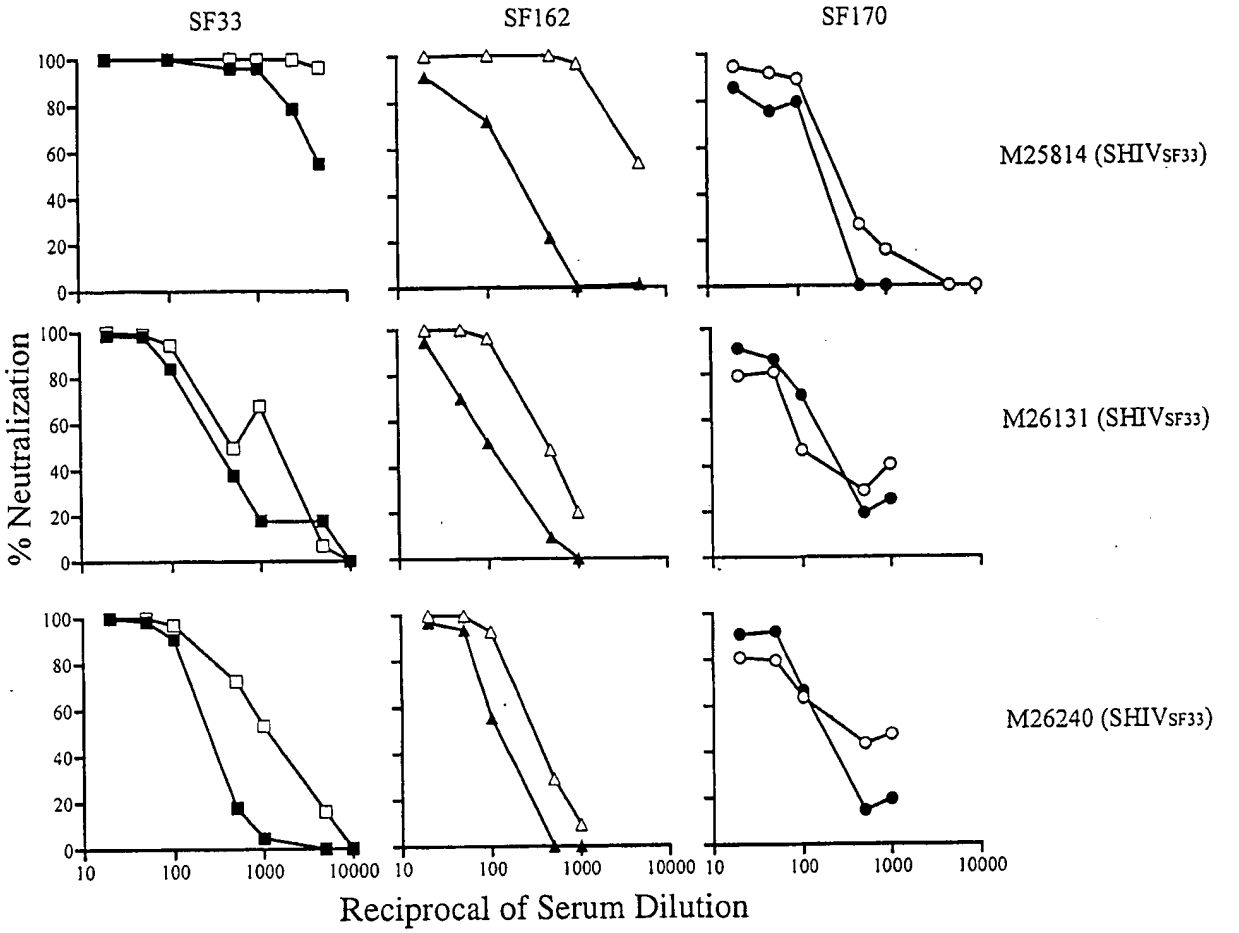

FIG. 2. Sera from macaques infected with $SHIV_{SF33}$ show broadly neutralizing activity. Neutralization of autologous and heterologous viruses by serial dilutions of sera from $SHIV_{SF33}$-infected animals M25814 (week 96), M26131 (week 53), and M26240 (week 53) is presented. Open and closed symbols indicate the absence and presence, respectively, of the V3 loop glycan of autologous SF33 (squares) and heterologous SF162 (triangles) and SF170 (circles) viruses. The neutralization profiles shown are representative of those from at least three independent experiments.

titers are comparable to or even slightly higher than those of the $SHIV_{SF33}$ M26131 and M26420 sera (Fig. 3 and Table 2). Only weak neutralizing activity was exhibited against both the HIV-1_{SF33} WT and V3T viruses by $SHIV_{SF162}$ sera M26419 and T528 (IC_{90} of 1:20 to 1:50), but the HIV-1_{SF170} viruses were resistant.

Increased exposure of conserved neutralization epitopes enhances their immunogenicity. Masking of the conserved neutralization epitopes by gp120 variable loops and carbohydrate moieties has been suggested to be partly responsible for their poor immunogenicity (62). The finding that sera from animals infected with viruses in which the conserved neutralization epitopes are more accessible (i.e., $SHIV_{SF33}$) exhibit substantial titers of cross-neutralizing antibodies suggests that increased exposure of these epitopes improves their immunogenicity. To build on this observation, the ability of $SHIV_{SF33}$ sera to neutralize a panel of HIV-1 Env pseudotyped viruses was examined. Furthermore, sera from animals infected with molecular clones of the neutralization-resistant pathogenic variant $SHIV_{SF33A}$, designated $SHIV_{SF33A.2}$ and $SHIV_{SF33A.5}$, were examined for their cross-neutralizing potential. The Env gp120s of $SHIV_{SF33}$ and pathogenic clones differed by over 25 amino acids, among which are glycosylation modifications in the V1, V2, and V3 domains (19).

We found that the M25814 $SHIV_{SF33}$ sera achieved 90% neutralization against viruses pseudotyped with TCLA, X4, or X4 R5 HIV-1 (HXB2, HIV-1_{SF2}, HIV-1_{SF13}, and HIV-1_{SF665}) as well as primary R5 HIV-1 (JRFL and ADA) Envs at serum dilutions of 1:20 to 1:50 (data not shown). No significant difference in neutralizing titers was observed for the X4 and R5 viruses, consistent with targeting of conserved functional epitopes of envelope glycoproteins. In contrast, sera from $SHIV_{SF33A.2}$- and $SHIV_{SF33A.5}$-infected animals displayed weak neutralizing titers, even against viruses that lack the V3 loop glycan (Fig. 4). Ninety percent neutralization was achieved only for the HIV-1_{SF33} Env-based viruses, with the V3 glycan-deficient WT virus being more sensitive (IC_{90} of 1:20 to 1:50). The lack of potent neutralization of the V3 glycan-deficient virus HIV-1_{SF162} V3T by $SHIV_{SF33A.2}$ and $SHIV_{SF33A.5}$ sera contrasts with the profile seen for neutralization by anti-HIV-1 human sera (Fig. 1). Determination of whether this reflects a difference in the antigenic structure of

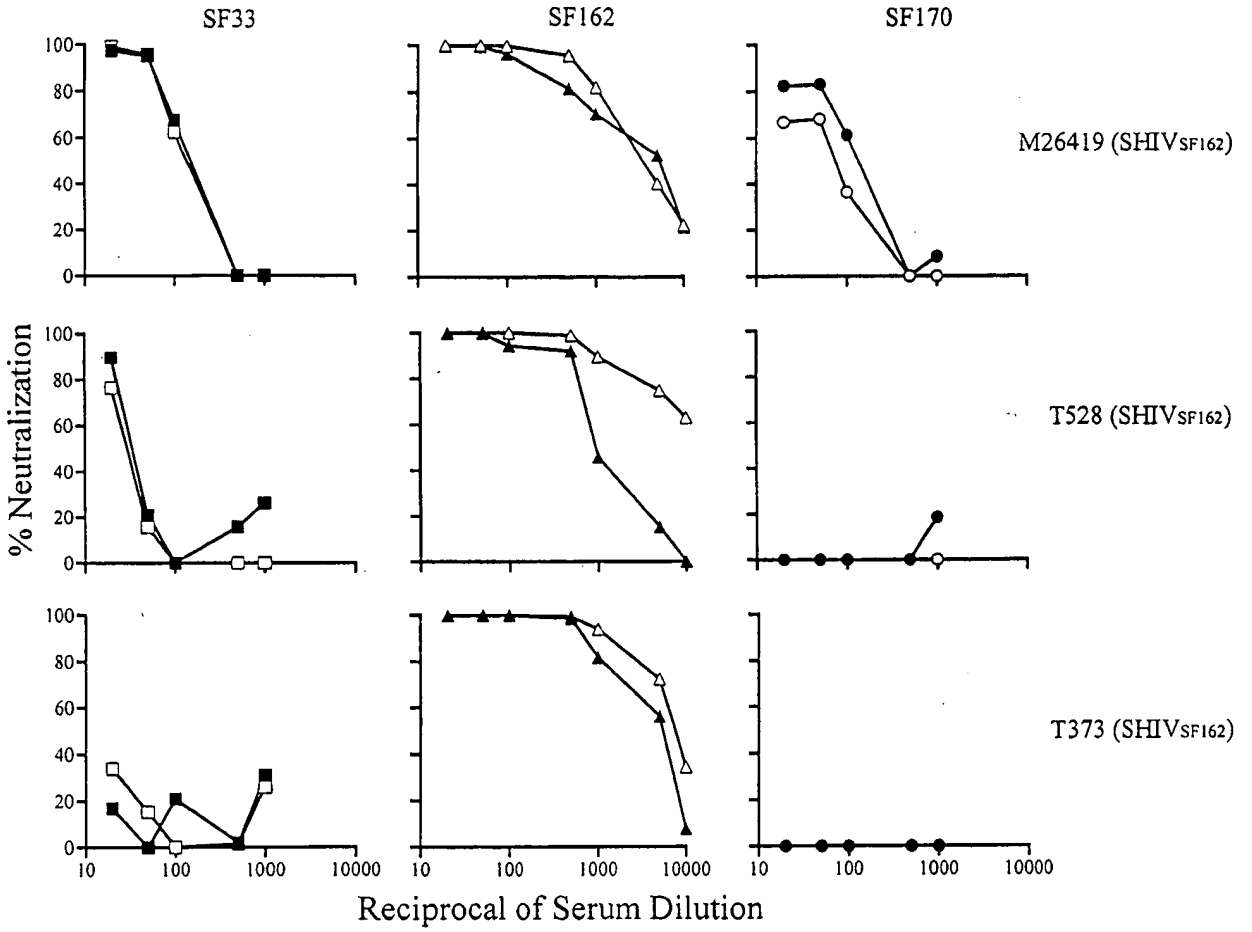

FIG. 3. High autologous but limited heterologous neutralizing antibody titers in sera from macaques infected with $SHIV_{SF162}$. Sera from M26419 (week 52), T528 (week 57), and M373 (week 79) were tested for their neutralizing activity in single-round infection assays as described in the text. Open and closed symbols indicate the absence and presence, respectively, of the V3 loop glycan of autologous SF162 (triangles) and heterologous SF33 (squares) and SF170 (circles) viruses. Results are representative of those from at least three independent experiments.

TABLE 3. Relative susceptibilities of WT and V3 glycan mutant viruses to neutralization with IgG CD4, anti-CD4BS, and anti-CD4i monoclonal antibodies

Virus	Neutralization[a] by:				
	IgG CD4	CD4BS monoclonal antibody:		CD4i monoclonal antibody:	
		IgG1 b12	F105	17b	48d
SF33 WT	++	++	−	++	+
SF33 V3T	++	+	−	+	±
SF162 WT	++	+	−	−	−
SF162 V3A	++	++	++	++	+
SF170 WT	−	−	−	−	−
SF170 V3A	±	±	−	−	−

[a] The extent of neutralization of V3 glycan-possessing and -deficient viruses by 5 μg of each monoclonal antibody per ml was determined as reported previously (32). ++, >90% neutralization; +, 50 to 90% neutralization; ±, 20 to 50% neutralization; −, <20% neutralization.

the X4, neutralization-resistant SF33A envelope and primary R5 envelopes that include $HIV\text{-}1_{SF162}$ or the impact of a pathogenic infection on the host immune response requires further investigation.

Broadly cross-reactive neutralization antibodies are detected early in a $SHIV_{SF33}$- infected macaque. It has been reported that compared to type-specific antibodies, a longer period of time is required for cross-neutralization antibodies to develop in infected humans and macaques (5, 38, 43). To investigate whether infection with a virus in which the conserved neutralization epitopes are more exposed shortens the time for antibodies directed against these sites to be developed, the cross-neutralization titers of sera collected from $SHIV_{SF33}$-infected macaque M25814 at 4, 8, 24, 32, 53, and 96 weeks postinfection (wpi) were determined. The results are summarized in Fig. 5. An overall increase in both homologous and heterologous neutralizing antibody titers over time in M25814 was observed, with serum collected at 96 wpi displaying the most potent cross-reactive neutralization titers (achieving 90% neutralization of the $HIV\text{-}1_{SF170}$ Env-based viruses at serum dilutions of 1:20 to 1:50). For

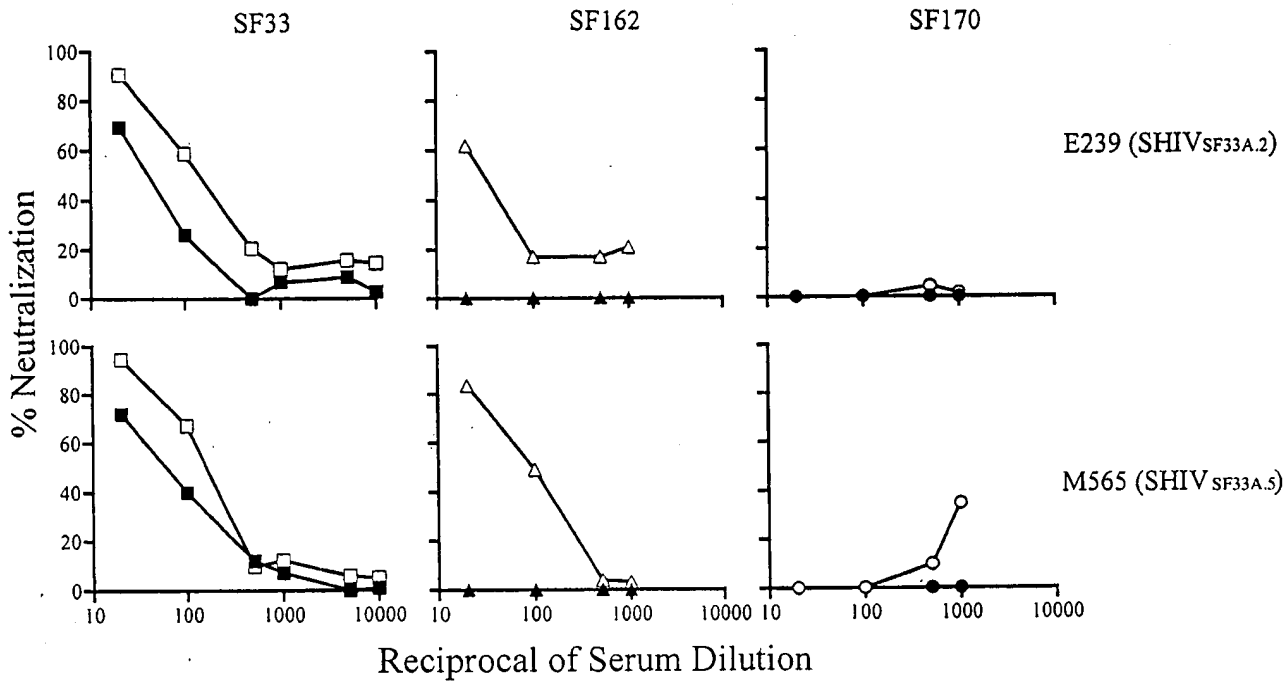

FIG. 4. Restricted neutralization of viruses by sera from two macaques infected with molecular clones derived from the pathogenic variant $SHIV_{SF33A}$. Open and closed symbols indicate the absence and presence, respectively, of the V3 loop glycan of SF33 (squares), SF162 (triangles), and SF170 (circles) viruses. The patterns shown are representative of those from at least three independent experiments.

the clade B viruses, neutralization antibodies against both homologous HIV-1_{SF33} WT and V3A viruses could be detected in serum collected as early as 4 wpi, with titers against the glycan-deficient WT virus being higher than those against the glycan-possessing mutant virus. Neutralization against the heterologous glycan-lacking HIV-1_{SF162} V3T virus could also be detected with week 4 M25814 serum, but the glycan-containing HIV-1_{SF162} WT virus was resistant. The latter virus was neutralized by sera collected from M25814 only after 32 wpi, with no significant increase in cross-neutralizing titers developing in this animal thereafter. These data suggest that antibodies directed against CD4BS and CD4i are present as early as 4 wpi in this $SHIV_{SF33}$-infected animal but that neutralization against glycan-possessing heterologous viruses requires a longer time to develop, perhaps reflecting the time required for such antibodies to mature and gain significant avidity (12, 13, 38, 48).

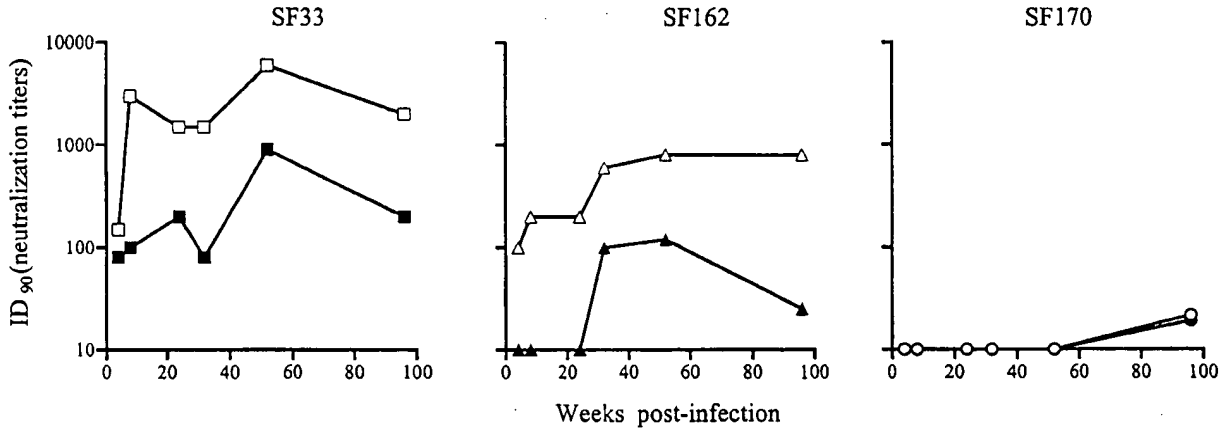

FIG. 5. Autologous and heterologous neutralizing titers of sera from $SHIV_{SF33}$-infected macaque M25814 collected over the time course of infection. Sera collected at 0, 4, 24, 32, 53, and 96 wpi were used. The serum dilution giving 90% neutralization (ID_{90}) of infection by autologous SF33 (squares) and heterologous SF162 (triangles) and SF170 (circles) viruses is shown. Open and closed symbols indicate the absence and presence, respectively, of the V3 loop glycan. The data are representative of those from at least three independent experiments.

DISCUSSION

In this study, we aimed to compare the immune responses to the HIV-1 envelope in humans and macaques by assessing the ability of HIV-1 and SHIV antisera to neutralize isogenic V3 loop glycan-possessing and -deficient viruses. This approach stems from our recent observation that the highly conserved N-linked glycan located at the N terminus of the V3 loop modulates the structure of the V3 loop, as well as blocking access to the conserved functional CD4BS and CD4i sites of gp120 (32). We reason that a similarity in the degree to which human and macaque antibodies recognize the V3 loop envelope variants would be indicative of a similarity in antigenic recognition of the HIV-1 envelope by the two hosts. Furthermore, an increase in the ability of heterologous HIV-1 and SHIV sera to neutralize viruses that lack the V3 loop glycan would be interpreted as neutralization mediated by antibodies directed at the CD4BS and CD4i sites present in these sera. Our findings that the presence or absence of the V3 loop glycan affects, to similar extents, recognition of the virus by polyclonal HIV and SHIV antisera illustrate that the ability of the macaque immune system to recognize HIV-1 gp120 is comparable to that of humans. Thus, it is reasonable to assume that data on the relative efficacies of HIV-1 Env-based vaccines generated in the SHIV-macaque model will be applicable to the human setting. Furthermore, the observations that the V3 loop glycan-deficient viruses are consistently more sensitive to neutralization by polyclonal human anti-HIV sera and sera from animals infected with viruses that have the glycan (i.e., $SHIV_{SF162}$ and $SHIV_{SF33A}$) indicate that the CD4BS and CD4i epitopes shielded by the V3 glycan are immunogenic in humans and in macaques.

An increase, compared to their glycan-containing counterparts, in susceptibility to neutralization of the clade B V3 loop glycan-deficient viruses, independent of coreceptor usage, by HIV sera is observed. A modest (two- to threefold) degree of difference in neutralization susceptibility is observed for the clade B TCLA, X4 HIV-1_{SF33} WT versus the V3T viruses, but a more dramatic difference (10- to 20-fold) is noted for the primary clade B, R5 HIV-1_{SF162} WT versus the V3A viruses (Fig. 1). This is true for neutralization with both clade B and clade A sera. Although broadly neutralizing anti-V3 antibodies have been described (16, 42), they are few in number. Cross-neutralization by sera from infected individuals, therefore, is attributed principally to antibodies against conserved conformational CD4BS and CD4i epitopes (41, 58, 63). We previously observed (32), and have summarized in Table 3, that the absence of the V3 loop glycan on HIV-1_{SF162} Env conferred greater susceptibility to neutralization with monoclonal antibodies directed against these sites than for HIV-1_{SF33} Env when compared to their glycan-possessing counterparts. In other words, the conserved neutralizing epitopes are better exposed in the absence of the V3 loop glycan on HIV-1_{SF162} than on HIV-1_{SF33}. The difference in the degree of neutralization susceptibility of HIV-1_{SF33} and HIV-1_{SF33} V3T compared to the HIV-1_{SF162} and HIV-1_{SF162} V3A viruses with HIV sera noted here correlates with the extent to which the V3 loop glycan affects the exposure of the CD4BS and CD4i sites on HIV-1_{SF33} and HIV-1_{SF162}. The finding that both the clade A glycan-possessing HIV-1_{SF170} and its V3 glycan-deficient variant are relatively resistant to neutralization with HIV-1 sera is also consistent with the rank order of the extent of CD4BS and CD4i epitope exposure. Neither of these viruses is very susceptible to neutralization with CD4BS and CD4i site antibodies (Table 3) (32). Collectively, our data are in agreement with previous findings (21, 54) and indicate that the conserved neutralizing epitopes are seen by the immune system in spite of their cryptic nature and that antibodies directed against these sites are prevalent in HIV-1-infected individuals.

The conserved gp120 neutralizing epitopes are also immunogenic in macaques. Similar to infection with HIV-1 in humans, infection with SHIV can be envisioned as immunization with different forms of HIV-1 envelope glycoproteins. We found that the presence or absence of the V3 loop glycan affected recognition of the virus by heterologous SHIV sera to the same extent as by human sera. That is, there is little difference in neutralization susceptibility of HIV-1_{SF33} compared to its glycan-containing variant V3T, as opposed to the more dramatic 10- to 20-fold difference in neutralization susceptibilities of HIV-1_{SF162} WT and V3A viruses. Thus, although it is generally believed that conserved neutralization epitopes of gp120 are poorly immunogenic, our findings suggest otherwise. High titers of neutralizing antibodies directed against the CD4BS and CD4i epitopes can be induced during natural infection of humans and macaques. The lack of neutralization or poor neutralization of primary isolates by HIV and SHIV sera therefore is a consequence of the many ways, including masking by glycosylation, by which the virus can escape immune recognition rather than of the lack of neutralizing antibodies. In this regard, it is interesting that all $SHIV_{SF33}$ sera, regardless of their gp120 ELISA titers, neutralized the heterologous HIV-1_{SF162} WT at 1:40 but displayed different neutralization titers against the glycan-deficient HIV-1_{SF162} V3A virus (IC_{90} of 1:2,000 for M25814 serum versus 1:200 for the 26240 and 26131 sera) (Fig. 2). This implies that a 10-fold increase in neutralization titers against the conserved epitopes is still insufficient to overcome the block to access to these sites on the glycan-containing virus.

Of the macaque sera tested, only sera from $SHIV_{SF33}$-infected animals exhibited broad neutralizing activity. The inability of the $SHIV_{SF162}$ sera to potently inhibit replication of the TCLA X4 $SHIV_{SF33}$ WT and V3T viruses (Fig. 3) contrasts with the ability seen for polyclonal HIV-1 sera (Fig. 1), raising the possibility that broadly cross-neutralizing antibodies may not be as prevalent in infected macaques as in humans. Nevertheless, it is important to recognize that the anti-gp120 ELISA titers of $SHIV_{SF162}$ sera are significantly lower than those of the HIV-1 sera tested (Table 2) and that the human sera were collected from individuals who have been infected for much longer periods of time (over 6 years). A comparison of the neutralizing ability of sera from recently seroconverted individuals to that of the $SHIV_{SF162}$ sera will be required to more fully compare the prevalences of antibodies directed against the conserved neutralizing sites in humans and macaques.

Despite the fact that sera from only a small number of infected animals were examined, a picture emerges in which chimeric virus containing the envelope of the TCLA, X4 HIV-1_{SF33} strain is able to elicit more potent cross-neutralizing antibodies than that containing envelopes of primary-like vi-

ruses (i.e., $SHIV_{SF162}$ and molecular clones of $SHIV_{SF33A}$). This finding is unlikely to be due to differences in the amounts of $SHIV_{SF33}$, $SHIV_{SF162}$, and $SHIV_{SF33A}$ antigens produced during infection, since pathogenic $SHIV_{SF33A}$ replicated to higher levels than $SHIV_{SF33}$ and $SHIV_{SF162}$ (data not shown) and yet was unable to elicit broadly cross-reactive antibodies. Montefiori et al. have also reported that monkeys infected with TCLA $SHIV_{HXB2}$, but not those infected with the primary isolate-derived dual-tropic $SHIV_{89.6}$ and $SHIV_{89.6PD}$, developed heterologous neutralizing antibody (37). It has been suggested that the gp120 conformation of TCLA viruses is biased towards an "open" state in which there is less masking of epitopes, including CD4BS and CD4i, for neutralizing antibodies (26, 32, 39, 62). This could account for the presence of higher titers of cross-neutralizing antibodies in monkeys infected with TCLA SHIVs. Nevertheless, we argue that increased exposure of conserved neutralizing epitopes on $SHIV_{SF33}$ Env gp120 as a result of the absence of the V3 loop glycan and/or other envelope modifications further contributes to its enhanced immunogenicity. Our finding that the broadly cross-reactive neutralization antibodies in $SHIV_{SF33}$-infected macaques can be detected much earlier (Fig. 5) and are of greater potency than has been previously reported for HIV-1-infected patients and for $SHIV_{HXB2}$-infected animals supports this argument. Indeed, macaques infected with variants of SIVmac239 mutated to lack N-linked carbohydrates in and around the V1 and V2 loops have been reported to generate antibodies that neutralize the fully glycosylated parent virus better than homologous sera (47). To directly address the effect of carbohydrate on the immunogenicity of HIV-1 envelope gp120 in infected macaques independent of other factors such as replication rates and cytopathicity, however, studies with site-directed glycan-deficient nonreplicating immunogens (e.g., SF33 V3T envelopes) will be required.

In summary, our findings suggest that the targets for neutralizing antibodies to HIV-1 envelopes raised in humans and macaques are similar and that conserved neutralization epitopes of HIV-1 Env gp120 are immunogenic in both hosts. Furthermore, our data support the use of modified envelope glycoproteins, such as the V3 deglycosylated form represented by $SHIV_{SF33}$ Env gp120, in which conserved epitopes are more exposed, as immunogens in various vaccine designs and strategies. The challenge, however, will lie in generating antibodies with sufficient titers and potency that are able to access the cryptic neutralization epitopes on primary isolates.

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